

Bio-oxidation of Terpenes: An Approach for the Flavor Industry

Juliano Lemos Bicas,* Ana Paula Dionísio, and Gláucia Maria Pastore

Laboratório de Bioaromas, Departamento de Ciência de Alimentos, FEA – UNICAMP, Rua Monteiro Lobato, 80 Campinas – SP, Brasil, CEP: 13083-862, Caixa Postal 6121

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1. Introduction

The terpenes are secondary metabolites of plants that are produced, in part, as a defense against microorganisms and insects in addition to their pollinator-attractive properties.¹ In mammals, terpenes contribute to stabilizing cell membranes, participate in metabolic pathways, and act as regulators in some enzymatic reactions.² Members of this class of chemicals have carbon structures which can be decomposed into isoprene (C₅H₈) residues and are classified, based on the number of carbons in the molecule, as monoterpenes (ten carbons), sesquiterpenes (fifteen carbons), diterpenes (twenty carbons), triterpenes (thirty carbons), and tetraterpenes or carotenes (forty carbons).³ The simpler terpenes (mono- and sesquiterpenes) are the major constituents of essential oils and are widely used in the perfumery industry, while di- and triterpenes are less volatile and are obtained from plant gums and resins.⁴ Carotenes are synthesized by bacteria, algae, fungi, and green plants and comprise more than 600 known structures.⁵ The most important terpenes and their oxygenated derivatives (terpenoids) cited in this study may be seen in Figures 1–3.

Terpenes are a good starting material for the synthesis of many fine chemicals due to their similar carbon skeleton. *R*-(+)-Limonene (**2**), for example, is the most abundant monocyclic monoterpene in nature, and it represents more than 90% of the orange peel oil; thus, it is an inexpensive precursor.^{6,7} The oxygenated derivatives of limonene, *e.g.* carveol (**24**), carvone (**25**), perillyl alcohol (**26**), menthol (**39**), and α -terpineol (**29**), are recognized for their pleasant fragrances,⁸ and some of them also present bioactivity against certain types of tumor cells, not only preventing the formation or progression of cancer but also regressing existing malig-

nant tumors.^{9,10} α -(**6**) and β -pinene (**7**), in turn, are found in high concentrations in turpentine, a paper and pulp industry residue, and they are, therefore, also available in bulk at a low price. These bicyclic monoterpenes are used as a fragrance substance that is used to improve the odor of industrial products and are also precursors of important flavor compounds, such as terpineols, borneol (**45**), camphor (**46**), citronellol (**11**), geraniol (**14**), menthol (**39**), verbenol (**48**), and verbenone (**49**).^{6,7} The tetraterpene β -carotene (**62**), an orange pigment found mainly in tropical vegetables, is a precursor of norisoprenoid ionones, molecules responsible for desirable fruity and floral flavors.^{7,11} Volatile carotenoid breakdown products have been long known as important flavor compounds.¹²

Of the approximately 6,500 known flavors, only 300 are commonly used. At present, 50 to 100 are produced by microbial fermentation, while the rest are mainly obtained by chemical synthesis.¹³ The scientific literature contains many examples of reviews about the chemistry of monoterpenoids,¹⁴ the chemical reactions of terpenes to produce flavors¹⁵ and other fine chemicals,¹⁶ the biotransformation of limonene^{17,18} and other terpenes,^{2,19–21} and natural flavor production via biocatalysis.^{22–28} However, no reference was found of a paper that congregates all these subjects. This review discusses the methods developed until present days for terpene oxidation in the production of molecules that attract great interest by the flavor industry, especially the monoterpene and norisoprenoid natural flavor compounds produced *via* microbial biotransformation (bioflavors).

2. Chemical Transformations

One of the most extensively studied reactions involving olefins is nitroschlorination.²⁹ The first description of the transformation of terpenes using gaseous nitrosyl chloride was about 130 years ago.^{30,31} In the early 1950s, Royals and Horne Jr.³² applied the nitrosyl chloride method to produce *R*-(-)-carvone (**25**) as the sole product from *R*-(+)-limonene (**2**), with an overall yield of 56–60% (Figure 4). Years later, a similar procedure was followed for the preparation of carvone (**25**) from orange oil.³³ Some other terpene nitroschlorination processes and their variants have also been studied and patented.^{34–38} This is currently an industrially important methodology for the preparation of the flavor compound *R*-(-)-carvone, the main monoterpene of spearmint oil (70–80%), which has a herbaceous odor reminiscent of spearmint.⁷

Other widely investigated mechanisms for the allylic oxidation of olefins consist of the use of selenium dioxide,^{39–42} and many such terpene oxidation methods have been described in past years. In the case of limonene (**2**), the

* E-mail: jlbicas@gmail.com.



Juliano Lemos Bicas is a Food Engineer (State University of Campinas—Unicamp, Brazil) and concluded his Ph.D. at the Food Science Department (DCA, Unicamp) on March 2009 under the supervision of Prof. Dr. Gláucia Pastore. His work was based on the microbial biotransformation of monoterpene compounds for the production of bioflavors and the determination of their bioactivity. He has recently been to the Laboratoire de Génie Chimique et Biochimique (Université Blaise Pascal, France), where he developed part of his doctorate project under the cosupervision of Prof. Dr. Christian Larroche.



Ana Paula Dionísio received her Food Science degree with honors from the University of São Paulo (USP, Brazil) in 2006. She is working on her Ph.D. at the Food Science Department (DCA—Unicamp), supervised by Prof. Dr. Gláucia Pastore, and studied in the Laboratorio de Biotransformaciones Industriales (Universidad Complutense de Madrid, Spain). She is currently working with biotransformation of carotenoids for bioflavor production.

reaction carried out in ethanol leads to the formation of limonen-4-ol (**30**) as the main product as well as limonen-10-ol (**31**) and carveol (**24**) in minor amounts,^{43,44} while limonene-1,2-diol (**33**) was favored in the SeO₂–H₂O₂ system.^{45,46} Myrtenal (**51**), a spicy flavor compound, may be prepared from α -pinene (**6**) using SeO₂–V₂O₅,⁴⁷ reaching relatively high yields (>75%) and selectivities (>85%),⁴⁸ or using SeO₂ in ethanol,^{49,50} and the kinetics of this oxidation has been studied for the selenium dioxide–vanadium system.⁵¹ Selenium dioxide was also applied to induce the oxidation of β -pinene (**7**),^{52–56} camphene (**8**),^{57–59} and some sesquiterpenes.⁶⁰ However, the possible formation of selenium and organoselenides in these kinds of reactions represents a problem to be considered, since selenium compounds are exceedingly toxic. This might be one of the reasons why such terpene oxidation methods are now in disuse.

The metal-catalyzed oxidation of terpenes has been extensively studied and might be an option for producing



Gláucia M. Pastore concluded her Ph.D. in 1992 at the Food Science Department (State University of Campinas, Brazil). She just after carried out postdoctoral studies at the Ohio State University (USA, 1993). She is currently a full professor at the Department of Food Science (Unicamp), director of the Faculty of Food Engineering (Unicamp), vice president of the Brazilian Society of Food Science and Technology, a member of the International Union of Food Science and Technology (IUFoST) Governing Council, and a member of the International Academy of Food Science and Technology (IUFoST). She has supervised more than 25 Masters and more than 20 Ph.D. theses. Her main contributions to science are in the domain of food biochemistry, especially working with functional foods, bioflavor production, and enzymology. She has more than 80 articles and four book chapters in international publications.

oxygenated derivatives. The palladium-catalyzed oxidation of limonene (**2**) and α -(**6**) and β -pinene (**7**) occurs mainly at the allylic sites of the molecule, and the main products are generally carvyl derivatives (conversions varying from 80 to 95% and selectivities from 75 to 90%).^{61–64} In fact, the mechanism of such oxidations seems to be *via* the intermediate formation of π -allyl palladium complexes.⁶⁵

Allal et al.⁶⁶ evaluated the influence of the catalyst and the reaction conditions in the oxidation of α -pinene (**6**). The conclusion was that a dropwise addition of the oxidants (*t*-BuOOH or H₂O₂) is needed to maximize the yields and the system Cu/*t*-BuOOH/O₂/70 °C promoted the formation of verbenone (**49**) (100% conversion and 70% selectivity), while Pd/H₂O₂/70 °C yielded verbenol (**48**) more efficiently (98% conversion and 78% selectivity). These systems were also studied in the oxidation of limonene (**2**), 3-carene (**9**), and valencene (**57**), and the best results were obtained when using Cu/*t*-BuOOH/O₂, which, in the case of valencene (**57**), yielded nootkatone (**60**) with 100% conversion and 80% selectivity.⁶⁶ The oxidation of α -pinene (**6**) catalyzed by other metal compounds, using H₂O₂ as the oxidizing agent, has also been reported⁶⁷ (see also references cited). The same oxidizing agent and a metal complex biomimetic catalyst to metalloenzyme methane monooxygenase were used to oxidize limonene (**2**), α -(**6**), and β -pinenes (**7**). The main products obtained were, respectively, the ketones carvone (**25**), verbenone (**49**), and pinocarvone (**53**).⁶⁸

The oxidation of monoterpenes using metal(salen) complexes (Figure 5) as catalysts has been widely described in recent years, and it seemed to replace the traditional techniques for the chemical reactions. These catalytic systems might be considered as cytochrome P450 analogues, since they involve oxometallic species (M=O) *via* a rebound mechanism such as the metalloporphyrins.⁶⁹ When using relative catalyst concentrations of from 0.03 to 0.05 and iodosobenzene as the terminal oxidant, the conversion of limonene (**2**) reached 50–60%, and the selectivities observed for *cis*- and *trans*-limonene-1,2-oxide (**32**) were 30% and

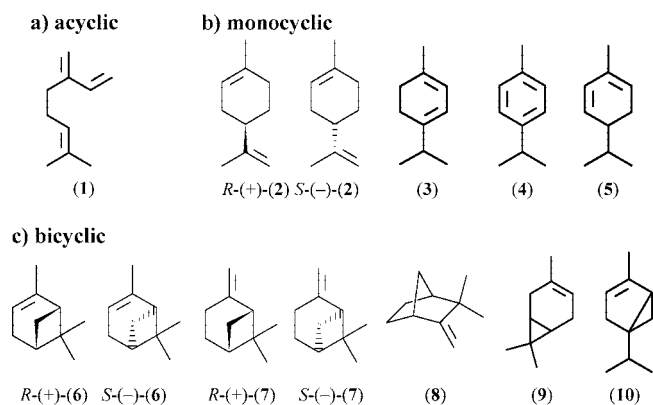


Figure 1. Main monoterpene hydrocarbons cited in this manuscript.

16.7%, respectively, 18.4% for carvone (**25**), and 10% for the two diastereoisomers of 1-*p*-menthen-9-al (**40**).⁷⁰ Under the same conditions, a conversion of 50–60% was observed for α -pinene (**6**), yielding, after 16 h of reaction, over 20% of a mixture of the corresponding epoxides (**55**) (55% selectivities) and between 2 and 6% of pinocampone (**54**) and myrtenol (**50**). For β -pinene (**7**) the optimal conversion (55%) was obtained after 4 h of reaction, with the maximum yield of myrtenal (**51**) isomers and epoxide (**56**) isomers varying from 6.5 to 23.2% and from 2 to 4%, respectively.⁶⁹ In a recent study, Lima et al.⁷¹ evaluated the main reaction parameters that could affect the allylic oxidation or epoxidation of the metal(salen)-catalyzed oxidation of limonene (**2**). The use of supercritical CO₂ instead of ordinary organic solvents was also investigated. In this system, the conversion was similar to that obtained in some organic solvents, but the higher selectivities (~40%) toward 1,2-epoxide (**32**) formation, as observed for organic solvents, only occurred after 4 h of reaction.⁷²

Other cytochrome P450-biomimetic chemical systems (generally based on metalloporphyrins) capable of carrying out alkane hydroxylation and alkene epoxidation have also been reported.^{73,74} In the specific case of terpenes, Skrobot et al.⁷⁵ showed the production of epoxides from these compounds. In an analogous work, the oxidation of monoterpenes by hydrogen peroxide catalyzed by porphyrins was also described.^{76,77} Other authors have made use of a photoexcited porphyrin to oxidize limonene (**2**) and produce a mixture of carvone (**25**) and another unknown product (with a mass spectrum similar to that of verbenone (**49**)) in concentrations of up to 3.4 g·L⁻¹ and 6.0 g·L⁻¹, respectively. However, the oxidative degradation instability of the metalloporphyrins and the difficulty of recovering this expensive catalyst limit their practical application. In this case one possible solution might be the immobilization of the metalloporphyrins on solid supports.⁷⁸

Photooxidations via singlet oxygen employing dyes as photosensitizers have increasingly attracted the interest of organic chemists for industrial scale production of flavor compounds. This green chemical approach is one attractive alternative to the traditional chemical synthesis, since it is a clean, traceless, and sustainable technology, although the high energy demand of most artificial sources is one challenge that must be overcome.⁷⁹ It has been employed in the production of ascaridole (**44**) from α -terpinene (**3**)⁸⁰ and in the oxidation of α -thujene (**10**)⁸¹ and others. The photooxidation of citronellol (**11**) for the production of rose oxide (**42**), an important fragrance used in the perfumery industry

(geranium perfumes), is the most distinguished example, a reaction currently performed industrially on a >100 tons per year scale by Symrise.⁷⁹ This reaction begins with the formation of two hydroperoxides in the presence of molecular oxygen, light, and a photosensitizer (usually rose Bengal or methylene blue), which are then reduced with Na₂SO₃ to the corresponding regioisomers alcohols; only one of them is converted by acid cyclization to form an epimeric mixture of rose oxides (Figure 6). Due to its industrial importance, this photoreaction is currently used as a prototype for comparison studies, being used as a model for the investigation of the reaction parameters in a photomicroreactor,⁸² for photoreactions under concentrated sunlight in the presence^{79,83} or absence of singlet oxygen,⁸⁴ and to study the use of ionically polymer bound photosensitizer.⁸⁵

For more detailed information on the chemical transformations of terpenes focused on the flavor industry, the reading of Swift¹⁵ and Monteiro and Veloso¹⁶ is recommended.

3. Biotransformation Processes

During recent years, there has been increasing pressure on the industries to adapt their processes and products to recent global tendencies. Environmental concern has forced the development of cleaner processes, according to the 3R rule (Reduce, Reuse, and Recycle), while the “dietetic revolution” imposes a growing demand for natural and, more recently, functional products containing the so-called bioactive compounds. In this context, biotransformation emerges as an attractive alternative for terpene oxidation, since, as compared to the traditional chemical methods, they proceed under mild conditions, have an elevated regio- and enantioselectivity, and do not generate toxic wastes, and the products obtained can be labeled as “natural”.^{23,26,28,86} In addition, the most significant strength of biotransformation processes is the ability to produce compounds not easily prepared by chemical methods.

Biotransformations can be briefly described as chemical reactions catalyzed by microorganisms or enzyme systems⁸⁷ and are usually carried out with growing cultures, previously grown cells, immobilized cells, purified enzymes, or multiphase systems.⁸⁶ According to De Carvalho and Da Fonseca,² 7% of the papers on terpene biotransformation published in the last ten years use purified enzymes as the biocatalyst, while plant cells, fungi, yeasts, and bacteria account for 11%, 33%, 2%, and 41%, respectively. In sequence, the (bio)oxidation of terpenes via biotransformation processes using isolated enzymes, whole plant cells, and microorganisms will be considered in detail.

3.1. Use of Purified Enzymes

The use of purified enzymes in bioconversions may be advantageous or necessary in some cases, such as (i) when the membrane of the intact cell prevents appropriate substrate or product permeation, (ii) when there is posterior product degradation or undesirable side reactions involving other enzymatic systems, (iii) when the enzyme of interest is excreted by the cell and might be easily purified from the medium after biomass removal, or (iv) when the enzyme of interest is commercially available. On the other hand, enzyme purification is often tedious, time-consuming, and expensive.⁸⁸

The enzyme-generated reactive oxygen species process is a method that combines chemical oxidation and the enzy-

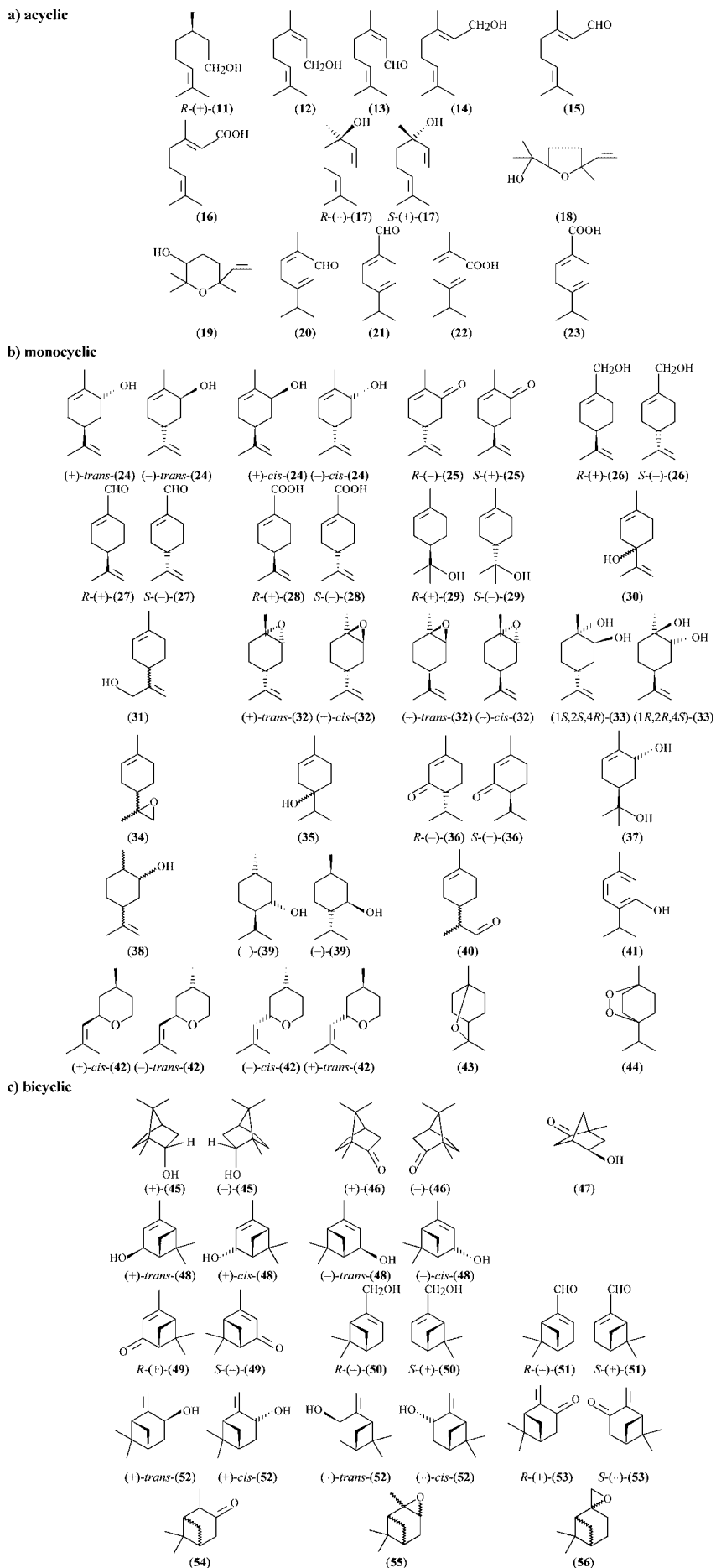
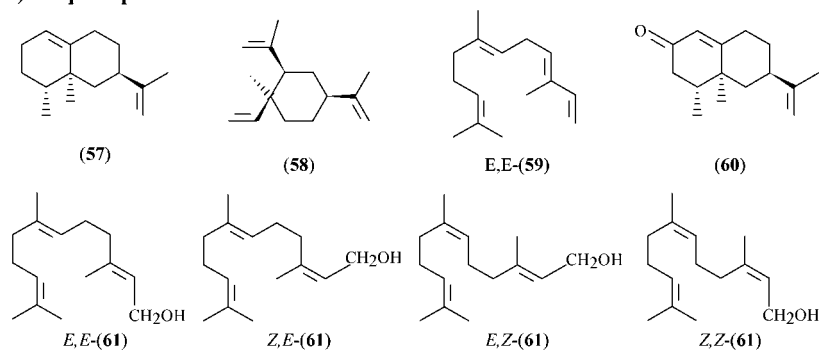
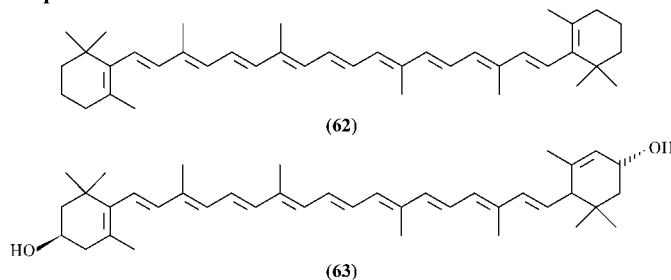


Figure 2. Main oxygenated monoterpenes cited in this manuscript.

a) Sesquiterpenes



b) Tetraterpenes



c) Degraded terpenoids

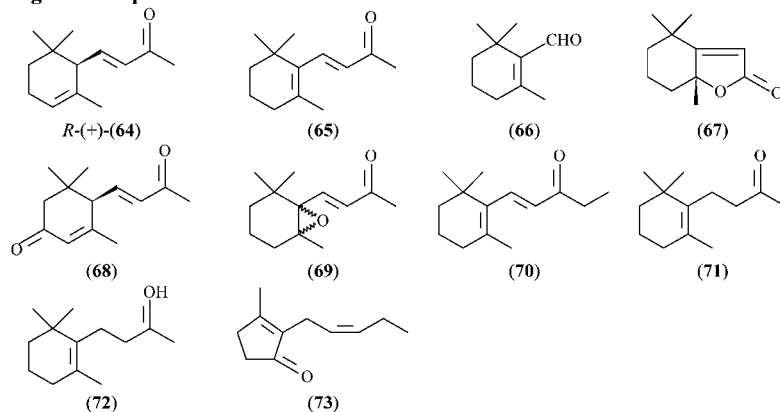


Figure 3. Other terpenes and oxygenated terpene-derived compounds cited in this study.

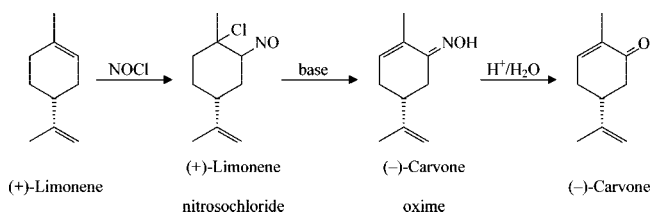


Figure 4. Oxidation of *R*-(+)-limonene to *R*-(-)-carvone by applying the nitrosyl chloride method: a commercially important reaction.⁷

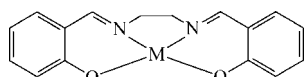


Figure 5. Basic structure of metal (salen) complexes.

matic production of the oxidizing agent. This system can be applied to the biotechnological production of the aroma compound β -ionone (**65**) from β -carotene (**62**) by the use of enzymes, *e.g.* lipoxygenase^{89,90} and xanthine oxidase,⁹¹ reaching concentrations not higher than $350 \mu\text{g}\cdot\text{L}^{-1}$. However, to be cleaved, the lipophilic substrate needs to be present in the aqueous phase where the enzymes work. An alternative to overcome this problem might be the use of micelles dispersed in water or solvent.⁹⁰

An enzymatic system developed by Trytek and Fiedurek⁹² is apparently less sensitive to substrate concentration and temperature variation when compared to the microbial transformation methods. In this study, the optimum medium conditions for the conversion of limonene (**2**) to carvone (**25**) (apart from other side products) using glucose oxidase and horseradish peroxidase were pH 7.0, 1.5% substrate, 50 °C, and a reaction time of 16–24 h. This work was of great scientific value, since it described an original method for biotransforming a monoterpene using cell-free enzymes. However, the yield obtained was too low ($<10 \text{ mg}\cdot\text{L}^{-1}$) for an industrial application; thus, enzyme immobilization techniques might be considered for future similar studies.

Horseradish peroxidase was also studied in the enzymatic oxidation of citronellol (**11**). This reaction occurs predominantly after double C–C linkage epoxidation reactions, followed by epoxide solvolysis.⁹³ Another peroxidase, present in the mycelium-free culture supernatant of the edible fungus *Lepista irina*, was able to degrade β -carotene (**62**), yielding flavor compounds. The degradation occurred most efficiently at 34 °C with a pH optimum between 3.5 and 4, and the main volatile breakdown products formed were β -ionone (**65**)

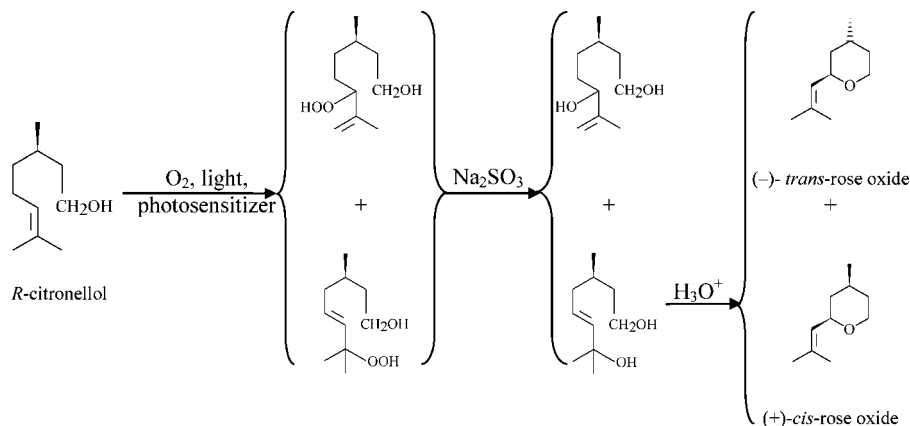


Figure 6. Photooxidation of citronellol for the production of rose oxide isomers.

(up to 12%), β -cyclocitral (**66**) (up to 2%), dihydroactinidiolide (**67**) (4%), and 2-hydroxy-2,6,6-trimethylcyclohexanone (6%).⁹⁴

Alcohol dehydrogenase can be used in the production of food additives, especially flavoring agents. One example is the oxidation of geraniol (**14**) to geranial (**15**) using horse liver alcohol dehydrogenase in biphasic mediums.⁹⁵ In the same paper, different organic solvents and three cofactor regenerating methods were studied. Other monoterpene oxidations catalyzed by alcohol dehydrogenase recovered from plants have been described elsewhere.^{96,97}

The investigation of sesquiterpene biosynthesis in chicory led to the characterization of a cytochrome P450 hydroxylase which was shown to hydroxylate β -elemene (**58**)⁹⁸ and in a further study was shown to be able to hydroxylate a range of other sesquiterpenes exogenous to the plant, mainly yielding the respective isopropenyl or isopropylidene alcohols in very low concentrations.⁹⁹

Many other terpene enzymatic transformations in cell free systems have been described, although they were basically focused on the isolation and characterization of microbial enzymes¹⁰⁰ or plant enzymes for the elucidation of the biosynthetic pathways involving volatile terpenoid formation in vegetables, especially monoterpenoids in *Mentha* leaves^{101–103} and norisoprenoids in quince,¹⁰⁴ star fruit,¹⁰⁵ or nectarines.¹¹

3.2. Use of Integer Cells

According to Duetz, Van Beilen, and Witholt,¹⁰⁶ there are four main reasons to use whole cells rather than purified enzymes: (i) apart from its simplicity and economy, the use of whole cells protects the enzyme from shear forces and might extend the enzyme activity half-life in a stirred bioreactor; (ii) the removal of an enzyme from a membrane environment often leads to complete or nearly complete loss of activity; (iii) cascades of enzymatic reactions may be too complicated to perform *in vitro* because of the number of enzymes, cofactors, and substrates involved; (iv) the stoichiometric consumption of cofactors during the enzymatic reaction or chain of reactions may make the use of whole cells attractive. Besides, when using whole cells, the addition of cofactors is not required.¹⁰⁷

3.2.1. Plant-Cultured Cells

Plant cell cultures exhibit a vast potential for the production of specific secondary metabolites and may be used to transform cheap and plentiful substances, such as industrial byproducts, into rare and expensive products.²⁶ Cytochrome

P450 oxygenases from certain vegetable cells are known for their ability to oxidize monoterpenoids during their biosynthesis. Hence, the use of these cells in the biotransformation of terpenes has been investigated in recent years.¹⁰⁸

The biotransformation capacity of culture suspensions of *Achillea millefolium* was investigated using different monoterpenes and a mixture of farnesol (**61**) isomers. Except for geraniol (**14**), the other substrates tested (borneol (**45**), menthol (**39**), thymol (**41**), and farnesols (**61**)) yielded less than 1 mg·L⁻¹ of products. The authors concluded that part of the substrates added and the biotransformation products were converted into and accumulated as the glycosylated forms.¹⁰⁹ *Nicotiana tabacum* and *Catharanthus roseus* were investigated in the biotransformation of 3-carene (**9**) and α -pinene (**6**),¹¹⁰ and cell culture suspensions of the last species were also tested in the conversion of *R*-(-)-piperitone (**36**), which was regioselectively hydroxylated at the 4- and 6-positions.¹¹¹ Cultured cells of *Caragana chamlagu* were able to convert α -(**64**) and β -ionone (**65**) into 3-oxo- α -ionone (**68**) and 5,6-oxi- β -ionone (**69**) with yields of 50% and 87%, respectively.¹¹² Further terpenic and nonterpenic substrates were tested in biotransformation assays using a culture suspension of *Peganum harmala*.¹¹³

Picea abies, from which the byproduct turpentine is collected after the thermomechanical pulping process, has been widely studied in the biooxidation of terpenes using plant cell cultures. Their cell culture suspensions were tested in the biotransformation of α -pinene (**6**),¹¹⁴ limonene (**2**), and β -pinene (**7**),¹¹⁵ and the main products obtained were, respectively, *trans*-verbenol (**48**), limonene-1,2-oxide (**32**), and *trans*-pinocarveol (**52**). α -Pinene (**6**) proved to be the fastest reacting substrate, but immobilization of the *Picea abies* cells decreased the transformation rate without influencing the composition of the products or their absolute configuration.¹¹⁶ Immobilized *Solanum aviculare* and *Dioscorea deltoidea* cells were also applied to oxidize (-)-limonene (**2**). However, in this case the attack was preferentially at position 6, yielding mainly *cis*- and *trans*-carveol (**24**) and carvone (**25**), all in concentrations close to 0.2 mg per g of cells.¹¹⁷

Despite great academic interest, the insufficient enzymatic activity and low yields obtained limit the application of plant cell cultures in industrial processes.

3.2.2. Fungi and Yeasts

The use of microorganisms in monoterpene biotransformation is relatively recent, dating from the late 1950s and

mid 1960s. Initially, the studies of microbial terpene bi-conversion were based on the discovery of the metabolic pathways through which the substrates were metabolized. In fact, this mechanistic data are essential for the bioprocess engineering in the case of bioflavors production. But, in the last few years, the research in this field has been based on the discovery of novel flavor compounds and on the optimization of the process conditions, although many papers still deal with the elucidation of terpene metabolism by microorganisms. The main microbial metabolic routes for limonene (**2**),^{18,118} α -(**6**) and β -pinenes (**7**),¹¹⁹ and others²¹ have been well revised recently.

In pioneering studies, a soil pseudomonad was used for the microbial degradation of camphor (**45**),^{120–122} limonene (**2**),^{123,124} α -(**6**) and β -pinenes (**7**),^{125,126} citronellol (**11**), farnesol (**61**), and others.¹²⁷ The fungal-mediated oxidation of terpenes was described in the same period, after an *Aspergillus niger* capable of metabolizing α -pinene (**6**) to oxygenated products was selected among different molds.¹²⁸ Substrate concentrations of 0.6% (v·v⁻¹), an 8 h reaction time, and a temperature range of 27–28 °C maximized the yields of the tree main metabolites (verbenol (**48**), verbenone (**49**), and *trans*-sobrerol (**37**)).¹²⁹ Subsequently, the same *A. niger* strain was investigated in the conversion of other mono- and sesquiterpenes.¹³⁰

Currently, *A. niger* is one of the most extensively studied fungal species involved in monoterpene biotransformation. Some of the parameters involved in the transformation of α -pinene (**6**) to verbenone (**49**) by an *A. niger* isolated from soil underneath citrus trees were optimized one-at-a-time. The optimal conditions were obtained when the microorganism was incubated for 6 h with 200 mg·L⁻¹ of substrate and 6 g·L⁻¹ of glucose in a sodium phosphate buffer at pH 7.0. Although the product formation increased, the yield remained low (328 mg·L⁻¹).¹³¹ *A. niger* ATCC 9462 was investigated for the conversion of (–)- α -pinene (**6**), (–)- β -pinene (**7**), and (+)-limonene (**2**), but only the second compound was transformed by this strain. The best results, about 4% conversion of (–)- β -pinene (**7**), were achieved when the substrate was supplemented in five subsequent additions as a 1:1 ethanol solution; the yield was unaffected by cell induction.¹³² The product obtained, α -terpineol (**29**), is one of the most frequently used and inexpensive fragrance substances, commonly applied in cosmetics and household products, which makes this process rather disadvantageous. The other five monoterpene substrates, i.e. (+)- and (–)-limonene (**2**), α -(**6**) and β -pinene (**7**), and camphor (**46**), were used for the microbial production of aromas and fragrances by *A. niger* IOC-3913. The study was carried out in a liquid medium (with growing cells, pregrown cells, and immobilized cells) or in a solid medium, with a substrate supply via the gas phase. (+)- and (–)-Limonene (**2**) were not metabolized by the strain tested, while verbenone (**49**) and α -terpineol (**29**) were the main products after the biotransformation of α -(**6**) and β -pinene (**7**), respectively.¹³³ Contrarily, another *A. niger* strain tested for the biotransformation of (+)- and (–)-limonene (**2**), (+)- and (–)- α -pinene (**6**), and (–)- β -pinene (**7**) gave only satisfactory results for (–)-limonene (**2**), yielding 18% of carveol (**24**) and 15% of dihydrocarveol (**38**).¹³⁴ Further papers have described the biotransformation of limonene (**2**) to perillyl alcohol (**26**)¹³⁵ and the conversion of linalool (**17**) to furanoid (**18**) and pyranoid linalool (**19**) oxides mediated by *A. niger*.¹³⁶

Larger terpenoid molecules have also been used in biocatalytic studies with *A. niger*. Mikami et al.¹³⁷ selected an *A. niger* strain capable of transforming β -ionone (**65**) and β -methylionone (**70**) into analogous tobacco-related aroma compounds. Another *A. niger* strain was found to be an efficient biocatalyst for a similar process, producing about 2.5 g·L⁻¹ hydroxyl and oxo derivatives from β -ionone (**65**) after 230 h of cultivation.¹³⁸ Later, the same strain was immobilized in calcium alginate beads due to the low aqueous solubility of the precursor, and the reaction was carried out in a two-phase liquid system. The best yield, 3.5 g·L⁻¹, was obtained after 400 h of reaction.¹³⁹ The physicochemical parameters of that system were analyzed elsewhere.¹⁴⁰ In their paper, very interesting for its originality, Krings et al.¹⁴¹ reported the screening of submerged microbial cultures able to oxifunctionalize the sesquiterpene α -farnesene (**59**). One culture, identified as *A. niger*, exhibited the most versatile and attractive flavor profile. The oxidation of the sesquiterpenes valencene (**57**) and nootkatone (**60**) could also be performed by a soil-isolated *A. niger* as well as by other fungal strains.¹⁴²

In addition, *Aspergillus* sp., i.e. *A. cellulosa*, was capable of converting both enantiomers and the racemate of limonene (**2**) into limonene-1,2-diol (**33**) as the main product.¹⁴³ In fact, some authors concluded that diols are common intermediates in the monoterpene metabolism of fungi.¹⁴⁴

Penicillium sp. is another fungal genera well documented in terpene biocatalysis. It was observed that the biotransformation of limonene (**2**) by *P. digitatum* occurred in the first instants of the log phase and that the bioconversion activity was expressively enhanced by the addition of substrate during the microbial growth.¹⁴⁵ Other publications have described the biotransformation of limonene (**2**) to α -terpineol (**29**) using immobilized *P. digitatum* cells¹⁴⁶ and have studied the effects of cosolvents in this conversion.^{147,148} The maximum α -terpineol production (~3.2 g·L⁻¹) occurred with sequential substrate feeding.¹⁴⁵ According to Agrawal and Joseph,¹⁴⁹ the culture conditions (age of the culture, pH, glucose concentration, and nitrogen source), the substrate concentration, the amount of biomass, the pH of the buffer, the temperature, and the incubation time taken for biotransformation of α -pinene (**6**) by a *Penicillium* sp. were found to be very critical for verbenone (**49**) formation. A 15-fold increase in product recovery was observed under the optimized conditions: 100 mL of a 0.05 M phosphate buffer pH 7.0 incubated at 30 °C for 6 h with 20 mg of substrate and 200 mg of fungal biomass, which was harvested after 18 h of growth at 30 °C and pH 5.75 in potato dextrose agar, supplemented with 1% glucose and 0.025% yeast extract.¹⁴⁹ Curiously, the biotransformation using spores of *P. italicum*¹⁵⁰ or *P. digitatum* ATCC 201167¹⁵¹ was feasible for, respectively, geraniol (**14**) and nerol (**12**) or citral (mixture of neral (**13**) and geranial (**15**)) and nerol (**12**), yielding 6-methyl-5-hepten-2-one. The pathway involved in this kind of transformation was subsequently studied.¹⁵²

Among various different mono- and sesquiterpenoids, transformations mediated by fungi, such as *Corynespora cassiicola* and *Diplodia gossypina*, Abraham et al.¹⁵³ described a well distinguished process of recovering good yields of (1*S*,2*S*,4*R*)-limonene-1,2-diol (**33**) from *R*-(+)-limonene (**2**) with continuous substrate feeding in a 100 L bioreactor filled with 70 L of culture medium. When 1300 g of substrate was used, 900 g of (1*S*,2*S*,4*R*)-limonene-1,2-diol (**33**) and small amounts of the (1*R*,2*R*,4*R*)-diastereoisomer were re-

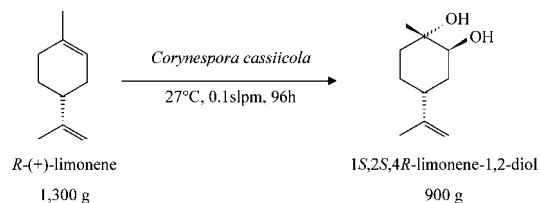


Figure 7. Economic way for preparing 1*S*,2*S*,4*R*-limonene-1,2-diol from *R*-(+)-limonene in a 100 L bioreactor.¹⁵³

covered after a 96 h process, representing an economic way of preparing diols (Figure 7). The psychrotrophic *Mortierella minutissima*¹⁵⁴ was also studied for the fungal conversion of limonene (**2**) into perillyl alcohol (**26**)/perillic acid (**28**), and the best results, approximately 120 mg of perillyl alcohol (**26**) per liter, were obtained after 120 h at 15 °C and pH 6.0. The authors concluded that the use of lower temperatures might reduce volatilization of the substrate and product, favoring the biotransformation process. Apparently, this is the only terpene biotransformation process applying a psychrotrophic microorganism. In another manuscript, it was shown that *Cladosporium* sp. could transform limonene to about 0.7 g·L⁻¹ of α -terpineol (**29**).¹⁵⁵ The same transformation was feasible, but with lower α -terpineol production (~0.4 g·L⁻¹), when an agroindustrial residue (cassava wastewater) was employed as an alternative culture medium for fungal cultivation, in this case a *Fusarium oxysporum* strain.¹⁵⁶ Using a similar technique, Maróstica Jr. and Pastore¹⁵⁷ noticed that *Penicillium* sp. was able to produce *cis* and *trans* rose oxides (**42**) from citronellol (**11**), such as *Cystoderma carcharias*.¹⁵⁸ The use of agroindustrial residues in bioprocesses seems to be a rising trend to overcome high manufacturing costs¹⁵⁹ (see also cited references), including the production of flavors.¹⁶⁰ Another alternative for the process optimization is the use of a statistical methodology (e.g., response surface methodology) to evaluate different parameters at the same time. This approach has employed production of α -terpineol (**29**)¹⁶¹ and other flavor compounds.¹⁶²

An interesting alternative to generate flavor compounds was via the fungal conversion of larger terpene molecules to volatile breakdown products. In this context, Zorn et al.¹⁶³ described an original method for screening microorganisms able to cleave β -carotene (**62**) to flavor compounds. From more than 50 filamentous fungi, ten bleached the zone surrounding the mycelium when grown in β -carotene-containing agar plates, suggesting the consumption of tetraterpene. Submerged cultures of four selected strains, *i.e.* *Ganoderma applanatum*, *Hypomyces odoratus*, *Kuehneromyces mutabilis*, and *Trametes suaveolens*, formed dihydroactinidiolide as the sole conversion product from β -carotene (**62**), while other carotenoid-derived volatile metabolites, mainly β -ionone (**65**), were detected in the mycelium-free culture supernatants from *Ischnoderma benzoinum*, *Marasmius scorodonius*, and *Trametes versicolor*.¹⁶³ A mixed culture formed by *Bacillus* sp. and *Geotrichum* sp. produced tobacco aroma compounds from lutein (**63**) after formation of the intermediate β -ionone (**65**). The second microorganism was responsible for the production of β -ionone (**65**), while the bacilli modified it to the aroma compounds 7,8-dihydro- β -ionone (**71**) and 7,8-dihydro- β -ionol (**72**).^{164,165} As already reported for *A. niger*, the filamentous fungus *Lasiodiplodia theobromae* ATCC 28570 may also metabolize the flavor compound β -ionone (**65**) to a complex mixture of metabolites reminding one of the tobacco flavor.¹⁶⁶ A similar hydroxy-

lation of α -(**64**) and β -ionone (**65**) at positions 3 and 4, respectively, was performed by selected strains of the bacteria *Streptomyces*. It was demonstrated that the transformation of α -ionone (**64**) proceeded with both high regio- and stereoselectivity.¹⁶⁷ Other fungal species, such as *Armillariella mella*¹⁶⁸ and *Botrytis cinerea*^{169,170} were able to biotransform, respectively, α -(**6**)/ β -pinenes (**7**) and a great variety of other terpenes.

Novel fungal strains are continuously being selected based on their ability to biotransform terpenes, and a promising alternative for screening potential fungi is the solid phase microextraction (SPME) technique to identify the biotransformation products, such as limonene-1,2-diol (**33**), α -terpineol (**29**), and the isomers of rose oxide (**42**), for both sporulated surface and submerged fungal cultures.^{171,172}

Interestingly, as far as we know, there are only a few descriptions of yeast-mediated terpene biotransformation processes. The yeast *Candida tropicalis* MTCC 230 has shown its capacity to oxidize α -pinene (**6**) to α -terpineol (**29**) with an overall yield of 77% after 96 h at 30 °C, when 0.5 g·L⁻¹ of substrate was used. The product concentration remained stable up to 120 h of reaction time.¹⁷³ In a recent manuscript, Pinheiro and Marsaioli¹⁷⁴ described the use of whole *Trichosporum cutaneum* cells in batch reactions to prepare oxiderivates of *cis*-jasmone (**73**), *R*-($-$)-carvone (**25**), α -(**64**) and β -ionones (**65**), and *R*-(+)-limonene (**2**). Other examples are the conversion of limonene (**2**), α -pinene (**6**), β -pinene (**7**), and some monoterpenoids by yeast or yeast-like fungi,^{175–177} and the modification of hop aroma terpenoids by ale and lager yeasts.¹⁷⁸ In this context, further descriptions of terpene biotransformations by yeasts would be of great scientific value.

3.2.3. Bacteria

Although the microorganism-mediated conversion of terpenes seems to proceed via cytochrome P450 monooxygenases,^{17,179,180} there are indications that the cytochrome P450 oxygenases of *A. niger* are not involved in the transformation of limonene (**2**) to perillyl alcohol (**26**).¹³⁵ The first step to find adequate biocatalysts is the screening of those solvent-resistant microorganisms that can use the substrate as sole carbon source.¹⁸¹ This indicates the existence of a substrate-degrading metabolic pathway, which can possibly accumulate interesting intermediate products.

Similar to the first studies, various pseudomonads have been applied to the biotransformation of terpenes.⁴ Members of this bacterial genus have shown good resistance to solvents,¹⁸² have the metabolic flexibility to grow in a wide range of organic compounds as the sole carbon source, and possess a wide variety of oxygenases and related enzymes for the activation and cleavage of terpene molecules.⁴ Yoo, Day, and Cadwallader¹⁸³ isolated a soil pseudomonad that could metabolize both α - (**6**) and β -pinenes (**7**), resisting concentrations of up to 10% of these terpenes. The possible pathway for the degradation of α -(**6**) and β -pinenes (**7**) by this pseudomonad was later described.¹⁸⁴ In earlier studies, some workers detected acid metabolites accumulated by *Pseudomonas* PX1¹⁸⁵ and *P. putida* PIN11¹⁸⁶ after the oxidation, followed by the ring cleavage of α -pinene (**6**), suggesting a different pathway from that determined in the above study. Years later, it was demonstrated that *P. putida* GS1 could convert limonene (**2**) solely to perillic acid (**28**), with this remaining stable in the culture medium.¹⁸⁷ In sequence, Mars et al.¹⁸⁸ concluded, after analyzing two *P.*

putida strains (GS1 e F1) and one recombinant *E. coli* strain, that the enzymes involved in this biocatalysis belonged to the *p*-cymene (**4**) degradation pathway. Another *P. putida* strain, MTCC 1072, has shown the ability to metabolize limonene (**2**), producing perillyl alcohol (**26**) and sobrerol (**37**), with yields of 36% and 44%, respectively.¹⁸⁹ Divyashree et al.¹³² described a *P. putida* isolate capable of biotransforming (+)- and (-)-limonene (**2**), (+)- and (-)- α -pinene (**6**), and (-)- β -pinene (**7**). The most important flavor compounds obtained in this study, i.e. verbenol (**48**), dihydrocarveol acetate, and verbenone (**49**) in yields of 35%, 20%, and 10%, respectively, resulted from the bioconversion of (+)- α -pinene (**6**). The other monoterpenes were metabolized into different oxidized products.

Other members of this genus have also been applied in the oxidation of monoterpenes. The ability of *Pseudomonas gladioli* to utilize limonene (**2**) as the sole carbon source was first described by Cadwallader et al.¹⁹⁰ The microorganism attacked the molecule at positions 7 and 8 to form perillic acid (**28**) and α -terpineol (**29**) ($1.0 \text{ g}\cdot\text{L}^{-1}$), respectively. The enzyme responsible for the α -terpineol (**29**) formation, an α -terpineol dehydratase, was further isolated and characterized.¹⁹¹ This enzyme converted stereoselectively and stereospecifically *R*-(+)-limonene (**2**) to *R*-(+)- α -terpineol (**29**).¹⁹² A soil-isolated bacteria identified as *P. maltophilia* was used to conduct the transformation of α -pinene (**6**) using resting cells or culture broth in a 30 L fermentor. The main natural products were identified as limonene (**2**), borneol (**45**), and camphor (**46**), while the acid fraction contained perillic acid (**28**) and 2-(4-methyl-3-cyclohexenylidene)propionic acid. Based on its O_2 uptake, it was demonstrated that this strain readily oxidized a diversity of monoterpenoids, e.g. β -pinene (**7**), limonene (**2**), α -phellandrene (**5**), 1,8-cineole (**43**), and others.¹⁹³ *Pseudomonas fluorescens* NCIMB 11671, a microorganism capable of completely degrading α -pinene (**6**) and being used as a sole carbon and energy source, initiated the α -pinene (**6**) metabolism by a NADH-dependent double bond epoxidation, followed by two ring cleavage by an energy- and cofactor-independent α -pinene oxide lyase to form two aldehydes (isonovalal (**20**) and novalal (**21**)). After their oxidation, the resulting acids (isonoallic acid (**22**) and novalic acid (**23**)) formed 3,4-dimethylpentanoic acid, which integrated the β -oxidation.¹⁹⁴ A similar pathway was also evidenced for *Nocardia* sp.^{195,196} Further reports suggest a different dynamic for this pathway, explaining the formation of novalal (**21**) by isomerization of isonovalal (**20**).^{197–199} The study of this metabolic route led to the development of an optimized method for isonovalal (**20**) production from α -pinene oxide (**55**) by *Pseudomonas rhodesiae* CIP 107491,²⁰⁰ reaching over $100 \text{ g}\cdot\text{L}^{-1}$ of the product with a yield close to 80%.²⁰¹ Mutants of the *P. fluorescens* wild strain unable to grow on α -pinene (**6**) and/or α -pinene oxide (**55**) were isolated after chemical mutagenesis, in order to explore alternative pathways and to serve as a basis for subsequent cloning studies. In this paper, the authors proposed possible alternative pathways for the metabolism of α -pinene (**6**).²⁰² Recently, *P. rhodesiae* CIP 107491 and *P. fluorescens* NCIMB 11671 have been screened for their ability to grow and bioconvert different terpene sources in biphasic medium. The results indicated that *P. rhodesiae* is a specialist for the bioconversion of the pinene family (α - (**6**) and β -pinenes (**7**)) while *P. fluorescens* could also metabolize limonene (**2**) in two ways, with the most profitable being the production of α -terpineol (**29**) in

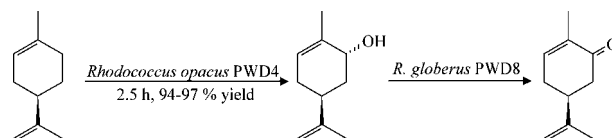


Figure 8. Biooxidation process of *R*-(+)-limonene to *trans*-carveol and carvone using *Rhodococcus* strains.²⁰⁵

concentrations of $\sim 11 \text{ g}\cdot\text{L}^{-1}$. Additionally, the cofactor-independent (bio)isomerization of β - (**7**) to α -pinene (**6**) was described for the first time.¹¹⁹

Some *Pseudomonas* species were tested for the biodegradation of acyclic monoterpenoids, and special attention was given to the use of the flowery-roselike flavor compounds citronellol (**11**) and geraniol (**14**) by *P. citronellolis*.²⁰³ One member of the last species, *P. citronellolis* DSM 50332, showed the ability to anaerobically degrade some monoterpenoids.²⁰⁴ This degradation can occur using a pathway earlier described for *P. citronellolis*.¹²⁵

Rhodococcus opacus PWD4 cells, which can use toluene as their sole carbon source, hydroxylated *R*-(+)-limonene (**2**) at position 6, forming enantiomerically pure *trans*-carveol (**24**). The maximal concentration of this product was obtained after 2.5 h, and the final yield was 94–97%. The posterior conversion of (+)-*trans*-carveol (**24**) into (+)-carvone (**25**) by *R. globerus* PWD8 illustrates that this strain might have a potential application in the industrial production of this ketone (Figure 8).²⁰⁵ Similarly, *R. erythropolis* DCL14 was able to transform (-)-*trans*-carveol (**24**) into (-)-carvone (**25**). In this case the use of a biphasic system improved the bioconversion rate,²⁰⁶ and this process has been shown to be feasible in a 3-L bioreactor.²⁰⁷ It is worth noting that, differently from the nitrosyl chloride methodology (Figure 4), this procedure does not involve a modification of the optical rotation. As shown for *P. fluorescens*,¹¹⁹ the same *R. erythropolis* DCL14 was also able to degrade *R*-(+)-limonene (**2**) initiated by a double bond epoxidation, forming (1*S*,2*S*,4*R*)-limonene-1,2-diol, (1*S*,4*R*)-1-hydroxy-2-oxolimonene, and (3*R*)-3-isopropenyl-6-oxoheptanoate. The opposite enantiomers ((1*R*,2*R*,4*S*)-limonene-1,2-diol, (1*R*,4*S*)-limonene-1-ol-2-one, and (3*S*)-3-isopropenyl-6-oxoheptanoate) accumulated when *S*-(-)-limonene (**2**) was employed as substrate, showing that the enzymes from this pathway are not stereoselective.¹¹⁸ Some reaction parameters involved in the biotransformation of geraniol (**14**) to geranic acid (**16**) by the *Rhodococcus* sp. strain GR3 were studied by Chatterjee,¹⁰⁷ who reported that the reaction occurred optimally at 30 °C and that the product concentration reached a maximum after 96 h and increased with an increase in the geraniol (**14**) concentration up to 1.0% (v·v⁻¹). A patent application describes the preparation of hydroxymethylated terpenes, more specifically perillyl alcohol (**26**), using the biotransformative capacity of a variety of bacteria, including members of the genus *Rhodococcus* sp.²⁰⁸

A *Xanthobacter* sp. isolated from river sediment, converted both enantiomers of limonene (**2**) into its 8,9-oxides ((4*R*,8*R*)-limonene oxide (**34**) was formed from *R*-(+)-limonene and a 78:22 mixture of (4*S*,8*R*)- and (4*S*,8*S*)-limonene oxide (**34**) from *S*-(-)-limonene) using cyclohexane as its sole carbon source, with the suggested involvement of a P450-dependent monooxygenase. The best results, $0.8 \text{ g}\cdot\text{L}^{-1}$ of epoxide, were achieved using 12 mM of substrate concentration.²⁰⁹ The endobacterium *Serratia marcescens* has shown the capacity for two specific transformations of α -pinene (**6**). In one of these biotransformations, the main product was *trans*-

verbenol (**48**) together with minor amounts of verbenone (**49**) and *trans*-sobrerol (**37**). Alterations in the culture conditions (use of another nitrogen source and the inclusion of glucose) changed the product profile, and in this case, α -terpineol (**29**) was the major product formed.²¹⁰

Some papers involving the *Bacillus* sp. metabolism of monoterpenes have also been published. In one of them, a strain isolated from pine trees, identified as *Bacillus pallidus* BR425, degraded α - (**6**) and β -pinene (**7**), as well as limonene (**2**). In the first case, significant amounts of pinocarveol (**52**), pinocarvone (**53**), carveol (**24**), carvone (**25**), and lesser amounts of myrtenol (**50**), myrtenal (**51**), limonene (**2**), and β -pinene (**7**) were recovered. Carveol (**24**) was a common metabolite for all the monoterpenes tested, suggesting that this compound, together with carvone (**25**), are central growth intermediates in BR425 pinene metabolism.²¹¹ In the same research field, Chang and Oriel²¹² isolated a thermophilic *Bacillus stearothermophilus* strain from orange peel that could use limonene (**2**) as the sole carbon source, converting it to perillyl alcohol (**26**) as the main product and α -terpineol (**29**) and perillyl aldehyde (**27**) as minor products. The same compounds were obtained, although not in the same proportions, when a 9.6 Kb chromosomal fragment was cloned and expressed in the recombinant *Escherichia coli*, which could grow on limonene (**2**) as its sole carbon source. However, the level of oxygenated monoterpenes recovered was considered insufficient for a possible industrial exploration of this process.²¹³ In a following study, Savithiry, Cheong, and Oriel²¹⁴ separated the limonene (**2**) hydration and methyl oxidation steps in the recombinant *E. coli* and noticed that a 3.8 Kb DNA fragment from the wild strain was responsible for growth on limonene (**2**) as the sole carbon source. It was later demonstrated that the use of limonene (**2**) as the sole carbon source by the recombinant *E. coli* resulted from the expression of a single gene, which codified a new monoterpene oxidative enzyme producing carveol (**24**), perillyl alcohol (**26**), and subsequently carvone (**25**) from limonene (**2**).²¹⁵

3.3. Use of Unconventional Biocatalysts

Although the greater part of the biooxidation processes described in the literature is performed by microorganisms, different unusual biocatalysts have been tested for the conversion of terpenes. Marine microorganisms, such as the cyanobacteria *Synechococcus* sp. PCC 7942, which could hydroxylate both *S*-(-)-limonene (**2**) and its oxide (**32**),²¹⁶ and the unicellular microalgae *Dunaliella tertiolecta*, which reduced aldehydes to the corresponding primary alcohols,²¹⁷ are interesting examples. Also, the unicellular microalgae *Oocystis pusilla* was capable of reforming a variety of monoterpenes. Although the main reactions were reductions of ketones to alcohols and of C=C double bonds, the formation of *trans*-carveol (**24**), carvone (**25**), and *trans*-limonene-1,2-oxide (**32**) from (+)-limonene (**2**) and of *trans*-pinocarveol (**52**) from (+)- β -pinene (**7**) was evidenced.²¹⁸ However, the most curious biocatalysts applied to terpene conversions are the superior organisms and animals.

Suggesting that the larvae of the cutworm *Spodoptera litura* possesses a high level of enzymatic activity against terpenoids, some authors have tested their biotransformation potential for limonene (**2**),²¹⁹ α -terpinene (**3**),²²⁰ β -myrcene (**1**),²²¹ terpinen-4-ol (**35**),²²² α -terpineol (**29**),²²³ menthol (**39**),²²⁴ camphor (**46**),²²⁵ geraniol (**14**),²²⁶ and others. The

terpene substrate (1–10 mg·g⁻¹, depending on the terpene tested) was mixed into the larvae's artificial diet, and the products were analyzed (GC-MS) in the organic extract of their frass (insect excrement). In general, the unsaturated monoterpenes were hydroxylated at the allylic position. In this case, the terpene metabolism was similar to the terpene metabolism in mammals. Actually, there are several examples of terpenoid oxidations by mammals, although they have not received much attention.^{227,228}

As may be observed, there are interesting underexplored ways to biotransform terpenes, since every superior organism with a well developed enzymatic system, especially those involved in xenobiotic metabolism, has the potential to oxidize these compounds. Further research in this field could have great scientific value, especially if it discovers new compounds, unknown metabolites with unique structures, potential biocatalysts, or original biotechniques.

4. Emergent Technology and Future Prospects

According to Leuenberger,⁸⁶ biotransformation might be a useful tool in organic chemistry, although some biotechnological developments are needed: optimization of the biocatalyst cultivation and biotransformation conditions (medium, temperature, agitation, pH, *etc.*), strain improvement by classical methods or by genetic engineering, development of an appropriate production facility with an efficient product isolation procedure, process simplification to minimize the manufacturing costs, and finally the scale-up. Moreover, some techniques, which in combination with conventional methods could contribute to cost reductions and render further industrial biotransformation processes feasible and attractive, might be applied: the use of immobilized cells, improving biocatalyst stability, and making a continuous production process possible; the use of biphasic media, increasing the solubility of the substrate, and avoiding its toxic effect toward the microorganism; and the use of recombinant DNA and protein engineering to improve the yields.⁸⁶ In this context, many scientists are in search of genetically modified organisms for a more effective terpene oxidation process.

Unspecific genetic modifications through induced mutation (colchicine, ethyl methanesulphonate, or ultraviolet radiation) of *Aspergillus* sp. and *Penicillium* sp., or protoplast fusion between members of these two genera was applied in order to improve verbenol (**48**) yields in the biotransformation of α -pinene (**6**).^{229,230} However, after the advent of DNA recombinant techniques, direct genetic approaches for increasing biotransformation rates and simplifying the process have been driving studies in this area.

Plant recombinant enzymes applied to hydroxylate *S*-limonene (**2**)²³¹ and to cleave carotenoids producing apocarotenoid flavor compounds²³² are already a reality. Additionally, some wild types and mutants of P450_{cam} and P450_{BM-3} have been investigated as a way to oxidize (+)-valencene (**57**) to (+)-nootkatone (**60**). The latter presented higher activity although less selectivity when compared to P450_{cam}.²³³ *Pseudomonas putida* P450_{cam}, which is known to convert (+)-camphor (**46**) to 5-*exo*-hydroxycamphor (**47**), was remodeled by designed mutagenesis, greatly enhancing activity for the oxidation of α -pinene (**6**) and *S*-limonene (**2**). The authors suggested that this technique could give rise to novel fragrances and flavorings or new biologically active compounds.²³⁴

Although the approach of cloning and the expression of terpene biotransforming genes have already been performed in *E. coli*,^{213–215} there are still only a few descriptions of the bio-oxidation of terpenes as carried out by cloned microorganisms, and the viability of such processes seems to be distant. One exception is the production of perillyl alcohol (**26**) from limonene (**2**) by *Pseudomonas putida*, expressing an alkene hydroxylase purified from *Mycobacterium* sp. HXN-1500. This process was performed in a 2-L bioreactor with a biphasic medium. After 75 h, the perillyl alcohol (**26**) accumulated in the organic phase reached 6.8 g·L⁻¹, equivalent to 2.3 g·L⁻¹ when calculated for the entire bioreactor contents.²³⁵ As claimed by the authors, this is a promising technique for the industrial production of this alcohol. Also, a very promising step forward toward a future industrial application of recombinant microorganisms for the industrial oxidation of terpenes was achieved with a recombinant *E. coli* expressing a variant of P450_{BM-3}. This strain was capable of biotransforming α -pinene (**6**) into α -pinene oxide (**55**), verbenol (**48**), and myrtenol (**50**) in an aqueous–organic two-phase bioprocess with a total product concentration of over 1 g·L⁻¹ after only 4 h.²³⁶

However, it is worth noting that the success of innovative flavor biosynthesis does not depend exclusively on genetically improved biocatalysts but also on process engineering, particularly when it comes to terpenoid flavor compounds.²⁷ As elsewhere stated,²³⁷ genetic engineering is expected to be a universal solution in the future; however, until then, a careful selection of strain associated with appropriate bioprocess engineering will remain essential to obtain high yield processes.

5. Conclusions

The flavor and fragrance industries have grown constantly with the growth in the world economy. In parallel, the chemical oxidation of terpenes for flavor synthesis tends to be gradually replaced by biotechnological methods, and the rising quest for natural sources of aroma compounds is forcing an adaptation of the manufacturing methodology. Moreover, the biotechnological approach described in this review could be particularly useful for biorefineries or industries that can recover, based on a building-blocks concept, bioactive compounds from industry waste and byproducts, resulting in a more sustainable flavor industry. Therefore, biotransformation processes, especially those applying filamentous fungi or bacteria, have arisen as a promising alternative. However, the low transformation rates and high production costs are still obstructing their wide-scale adoption. To overcome these problems, the genetic engineering technique seems to be a suitable choice, although such investigations are still in an embryonic stage. Therefore, more studies are essential to ensure the economical adoption of biotechnology for the production of flavor and fragrances.

The novel frontier in the field of food ingredients not only aims to provide good and economic technological applicability but is also part of the quest for functional ingredients and nutraceuticals that are directing scientific and technological development in this area. Hence, flavor terpenes produced *via* biotransformation are completely adapted to the new market demand, since, despite their natural nature, many of them have been proven to play an important biological role against certain types of cancer in *in vivo* studies.

6. Acknowledgments

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