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MINI-REVIEW

Biotransformation of α- and β-pinene into flavor compounds

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Abstract Products that bear the label "natural" have gained more attention in the marketplace. In this approach, the production of aroma compounds through biotransformation or bioconversion has been receiving more incentives in economic and research fields. Among the substrates used in these processes, terpenes can be highlighted for their versatility and low cost; some examples are limonene, α-pinene, and β-pinene. This work focused on the biotransformation of the two bicyclic monoterpenes, α-pinene and β-pinene; the use of different biocatalysts; the products obtained; and the conditions employed in the process.

Keywords Bioconversion · Aroma compounds · Volatile compounds · Natural aroma · Monoterpenes

Introduction

The field of food additives represents an important share of the world's economy and has been expanding rapidly. It has grown approximately 21.7% in the last 5 years, jumping from an annual turnover of US$ 29.94 billion in 2009 to US$ 36.45 billion in 2013 (Thomas 2014). Among all the food additives, the flavor and fragrance industries represent approximately 31% of this global market, moving about US$ 23.9 billion in 2013 (Thomas 2014; UBIC 2014). One of the main concerns of this industrial sector is to supply the increasing demand for "natural products" from consumers due to the awareness of the link between diet and health (Mapari et al. 2010). Therefore, the development of new processes and methods to obtain natural flavors and fragrances, including biotransformation, bioconversion, and enzymatic reactions, have been extensively studied (Maróstica and Pastore 2007a; Bicas et al. 2009).

European legislation defines "natural flavors" as chemical compounds with aroma properties, obtained from the raw material of animal or vegetable origin or by physical, enzymatic, or microbiological methods (Berger 2009). These products were usually obtained from higher plants through different methods of extraction (Akacha and Gargouri 2015). These natural compounds have a high value when compared with products obtained through chemical synthesis. For example, the natural extract of vanillin is commercialized for about US$4000/kg, whereas the synthetic product is sold for only US$12/kg (Krings and Berger 1998; Daugsch and Pastore 2005). Although it is advantageous economically, obtaining these compounds by extraction has limited potential for exploitation, since it is directly dependent of climatic factors and requires a large amount of raw material. These factors accentuate the ecological problems created by this process. In addition, the concentration of the product is generally not sufficient to supply the market demand. The vanilla extract only supplies 0.2% of the annual demand (Welsh 1994; Daugsch and Pastore 2005; Longo and Sanromán 2006; Bicas 2009; Akacha and Gargouri 2015).

The chemical synthesis of the desired product is a quick and cost-attractive alternative to overcome these problems of demand; however, chemical oxidation reactions can produce a racemic mixture composed by enantiomers and/or regioisomers with different sensory properties. This factor

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increases the complexity and the cost of the purification process (Brenna et al. 2003; Duetz et al. 2003; Daugsch and Pastore 2005; Longo and Sanromán 2006; Akacha and Gargouri 2015).

Obtaining aroma compounds through biotechnological processes emerges as a promising alternative because the fewer residues are generated, mild conditions can be used, and the regioselectivity and enantioselectivity to the substrate is better. For these reasons, this process can be characterized as being highly sustainable in accordance with the principles of "Green Chemistry" (Leuenberger 1990; Janssens et al. 1992; Anastas and Warner 1998; Serra et al. 2005; Berger 2009; Borges et al. 2009). Another relevant aspect is the fact that the products generated via biotechnology can be labeled as "natural" (Daugsch and Pastore 2005; Berger 2009).

The biotechnological process includes the biotransformation or bioconversion of specific substrates, as well as the de novo synthesis. In the latter case, the production of aroma compounds is performed using simple nutrients, like sugars, alcohols, and others, without the addition of special substrates. Therefore, the microorganisms utilize complex metabolic pathways, obtaining a mixture of various compounds as the final product. For example, there is the production of ethyl acetate by *Saccharomyces* sp. and lactic acid in fermented dairy products (Krings and Berger 1998; Vandamme and Soetaert 2002; Molina et al. 2012).

In the biotransformation process, the microorganism is induced to follow a specific metabolic pathway through the addition of precursors to the culture media. Thus, the substrate transformation takes place in a single step, or in two or more chemical reactions in the case of bioconversion, resulting in the formation of a specific product, such as in the production of compounds of economic interest from terpenes (Bicas et al. 2009). Another advantage of the biotechnological production of aroma is the possibility of using agro-industrial wastes as nutrient sources or wastes containing high concentration of substrates; for example, residues rich in terpenes generated in the processing of fruits and vegetables (Matthews and Braddock 1987; Pandey et al. 2000; Bauer et al. 2001; Yoo et al. 2001; Maróstica and Pastore 2007a; Bicas et al. 2009).

Terpenes are chemical compounds formed by isoprene units (C_5H_8) and are usually secondary metabolites from the defense mechanism of plants against pest and insects. They can also act as attractants for pollinators (De Carvalho and Da Fonseca 2006; Bicas et al. 2009). These compounds are also present in mammals and act in the stabilization of cell membranes, participation in metabolic pathways, and as regulators in enzymatic reactions (De Carvalho and Da Fonseca 2006; Bicas et al. 2009).

From an economic point of view, the abundance of terpenes in nature makes them a promising starting material for biotransformation and bioconversion processes (Molina et al. 2013). In addition to their economic importance, these compounds have become the subject of scientific interest because of their possible role in preventing diseases such as cancer (De Carvalho and Da Fonseca 2006; Bicas et al. 2009) and because of their antimicrobial (Bhatti et al. 2014) and antioxidant activities (Maróstica Junior et al. 2009). The monoterpenes and sesquiterpenes are the main constituents of essential oils (for example, *R*-(+)-limonene represents 90% of orange peel oil), and they are widely used in industry for the production of fragrances for cosmetics and cleaning products (Bicas et al. 2009). The monoterpene limonene is one of the most widely studied substrates in biotransformation processes (Molina et al. 2015), and a comprehensive review of the main routes to obtain limonene derivatives was presented by Duetz et al. (2003) and by Maróstica and Pastore (2007b). They compiled the most important information regarding the processes, biocatalysts, substrates, and strategies to obtain products by biotransformation, such as α-terpineol, perillyl alcohol, and limonenediol, among others (Duetz et al. 2003; Maróstica and Pastore 2007b).

α- and β-Pinene represent 75 to 90% of essential oils from conifers and can be found in concentrations in the range from 50 to 70% and from 15 to 30%, respectively, in turpentine, a byproduct of the paper and cellulose industry (Yoo and Day 2002; Lindmark-Henriksson 2003). These compounds are the most abundant bicyclic monoterpenes and can be precursors of aroma compounds of impact that are widely used in cosmetic and food industries, such as α-terpineol, verbenol, and verbenone (Bicas et al. 2009). Considering the fact that the Brazilian cellulose and paper sector uses about 72.5 million tons of wood (IBGE 2013), large volumes of turpentine are generated, and consequently, an inexpensive and abundant source of α- and β-pinene is available for studies of the bioconversion of these compounds (Maróstica 2006; Bicas et al. 2009; Bicas 2009).

Although α- and β-pinene are promising substrates, the physicochemical characteristics of these compounds represent challenges that require efforts for their effective application for biotransformation, such as their chemical instability, volatility, and toxicity to microbial cells (van der Werf et al. 1997). In addition, the low solubility of α- and β-pinene (0.026 and 0.049 mmol/L, respectively) results in a lower bioavailability of the substrate in submerged fermentation systems. The bioavailability can be improved by the addition of cosolvents to culture media to increase the mass transfer and the biotransformation rates (van der Werf et al. 1997; Tan and Day 1998). In addition, these compounds can be considered toxic to microorganisms, with log *P* values of 4.42 for α-pinene and 4.49 for β-pinene (values of log P between 1 and 5 are considered toxic to whole cells) (van der Werf et al. 1997). In spite of all these challenges, pinenes have been successfully used as substrates in biotransformation processes for the production of aroma compounds. Because of the potential for using these monoterpenes as substrates in biotransformation, the aim of

this review is to highlight the main microorganisms used for the biotechnological production of natural aroma compounds from α- and β-pinene, considering the abundance of these compounds, the economic importance of both substrate and products, and the commercial interest of the metabolites obtained.

Physicochemical and biological properties of pinenes

Figure 1 shows the structure of α- and β-pinene. Both structural isomers have enantiomers known in nature as (−)- and (+)-, resulting in four optically active isomers (Burdock 2010; Silva et al. 2012).

α–Pinene

α–Pinene is a colorless liquid, soluble in ethanol and oils and insoluble in water, with a boiling point of 155 °C. It has an intense and characteristic odor reminiscent of pine and turpentine. At concentration of 1%, it has a citrus and spicy, woody pine, and turpentine-like aroma. The threshold of detection is 2.5 to 62 ppb. The gustative threshold is 10 ppm, and it presents an intense, woody, piney taste with a notable camphor-like and turpentine taste (Burdock 2010).

The presence of one or more isomers of α-pinene was already reported in at least 400 essential oils, being found in higher concentrations in *Achillea millefolium*, *Artemisia tridentata*, Italian rosemary, wild thyme, French lavender, coriander, cumin, labdanum, neroli, lemon, and *Litsea cubeba* (Berger 2007). This terpene can be extracted directly from these sources or through the distillation of turpentine. Its utilization is mainly as a food addictive in the production of chewing gum and hard candy. The average annual consumption is 23,000 lbs (Burdock 2010).

Biological activities related to α-pinene and the essential oils wherein this compound is the principal component have been determined and demonstrate the importance of this monoterpene and its optical isomers as a promising candidate as a therapeutic agent. In a study performed by Chen et al. (2015), the antitumor activity of α–pinene was confirmed in cell lines and in animal models of hepatocellular carcinoma. The inhibitory effect was observed at a concentration of 8 mg/L for 72 h (in vitro) or 2 weeks (in vivo) (Chen et al. 2015). The ability to induce apoptosis and antimetastatic properties in vivo by α-pinene isolated from Aroeira vermelha (*Schinus terebinthifolius* Raddi (*Anacardiaceae*)) in a murine melanoma model was confirmed (Matsuo et al. 2011).

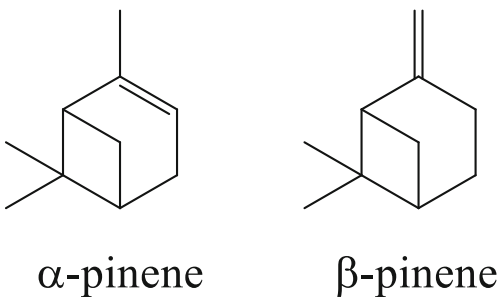

Fig. 1 Chemical structures of the α- and β-pinene: unsaturated bicyclic monoterpenes

Furthermore, a significant anti-leishmania activity against amastigote and promastigote forms of *Leishmania amazonensis* (protozoa of American cutaneous leishmaniasis) was recently demonstrated for both commercial α-pinene and the essential oil extracted from jambolan leaves (*Syzygium cumini*). This oil contains 87.12% monoterpenes, and of these, 31.85% consists of α-pinene (Dias et al. 2013; Rodrigues et al. 2015).

α-Pinene was also effective in improving the symptoms of allergic rhinitis in animal models. This monoterpene was immunobiochemically proven to be associated with the decrease in interleukin-4 (IL-4) levels in the spleen; suppression of migration of eosinophil and mast cells in injured tissues; and the inhibition of several factors, such as NF-kB, RIP2, and IKK-β, in addition to the activation of caspase-1 (Nam et al. 2014). The enantiomers (−)-α-pinene and (+)-α-pinene exhibit repellent properties against *Musca domestica* L. (*Diptera*: *Muscidae*), and the viability of using α-pinene as an additive to obtain a new repellent with more desirable properties than those of synthetic repellents has been proposed (Haselton et al. 2015).

Oxygenated derivatives of pinenes have also been reported to possess interesting biological properties. Antibacterial, acaricidal, and anti-inflammatory activities have been described for essential oils containing (1S)-(−)-verbenone (Bernardes et al. 2010; Kuo et al. 2011; Martinez-Velazquez et al. 2011). Recently, Scollard et al. (2016) demonstrated the antimicrobial potential of verbenone against the foodborne pathogen *Listeria monocytogenes* on fresh-cut lettuce, cantaloupe melon, and pineapple with modified atmospheres in model packages at 4 and 8 °C. In addition, its natural precursor, *cis*-verbenol, has been suggested to possess anti-ischemic, antioxidant, and anti-inflammatory activities that prevent neuronal cell death caused by oxygen-glucose deprivation and reduce cerebral ischemic injury (Choi et al. 2010).

Depending on the isomer, carvone has been shown to possess diverse in vivo biological activities, such as acaricidal, antihyperglycemic, antispasmodic, anticonvulsant, antimanic-like, and anxiolytic-like activities (Souza et al. 2013; Kang et al. 2015; Muruganathan et al. 2015; Peixoto et al. 2015; Nogoceke et al. 2016). Camphor and thujone, oxygenated derivatives that can be obtained from the biotransformation of pinenes, possess cytotoxic activity against colorectal cancer cell lines, and they enhance DNA repair mechanisms in in vitro tests at low concentrations (Nikolić et al. 2015).

β-Pinene

β-Pinene (Fig. 1) is a colorless liquid, soluble in oils and insoluble in water and ethanol, with a boiling point between 163 and 166 °C. At a concentration of 10%, the aroma characteristics are cooling, woody, piney, and turpentine, with traces of fresh mint, eucalyptus, and camphor. It can be detected at a threshold of 140 ppb. As for its taste, it has a characteristically fresh, pine, woody, and resinous taste at 15 to 100 ppm and a slight nuance of spicy, mint, and camphor (Burdock 2010).

Commercially, this compound is obtained from the distillation of American turpentine or through conversion of α-pinene (Bauer et al. 2001; Berger 2007). Generally, it is found along with α-pinene, but in lower concentrations (Bauer et al. 2001). In higher concentrations, the *d*- and *l*- forms have been found in the essential oils from several species of the genera *Artemisae* and *Cupressaceae*, also known as cumin and coriander, and the *l*- form is a constituent of several citric essential oils (Burdock 2010).

β-Pinene is an important intermediate in the production of citral, citronellol, geraniol, citronellal, linalool, ionones, and menthol, being mainly applied in bakery products, candy, and chilled dairy products. The average consumption is about 7800 lbs a year (Burdock 2010).

Most studies describe the antimicrobial properties of β-pinene, either purified or as part of essential oils (Leite et al. 2007; Gavrilov et al. 2010; Silva et al. 2012). Furthermore, two studies have recently demonstrated the antidepressant effect of β-pinene and the manner in which this monoterpene acts through an interaction with the serotonergic pathway, via 5-HT_{1A} receptors and with the dopaminergic system via D_1 receptors (Guzman-Gutierrez et al. 2012; Guzman-Gutierrez et al. 2015). β-Pinene is usually found in lower percentages than is α-pinene in several essential oils, and this fact might corroborate the fact that few studies explore their biological activities. Their possible applications are thereby neglected (Alvarez-Castellenos et al. 2001; Bajpai et al. 2007; Rajeswara Rao et al. 2011).

Biotransformation and bioconversion of α- and β-pinene

The biotransformation of the α- and β-pinene has been performed since the 1950s to obtain compounds of interest for cosmetics and cleaning product industries. The oldest reports describe only the biotransformation of α-pinene, for example, the use of *Aspergillus niger* for the production of *d*-verbenone, *d-cis*-verbenol, and *d-trans*-sobrerol (Prema and Bhattacharyya 1962). *Serratia marcescens* has also been used for the oxidation of α-pinene for the production of *trans*-verbenol (majority compound), verbenone, and *trans*-sobrerol (Wright et al. 1986).

In the past years, many improvements have been achieved in the biotransformation and bioconversion of these compounds, such as the production of significant amounts of isonovalal (about 16 g/L) by bacteria of the *Pseudomonas* genera (Zorn et al. 2004). However, there is still a lack of information regarding some parameters in the microbial transformation, such as the metabolic pathways involved and the dependence on enzymatic cofactors for the reactions (e.g., aldehyde dehydrogenase would be NAD^+ dependent) (Linares et al. 2009).

A variety of compounds can be obtained from the biotransformation of pinenes. Figure 2 shows some of the metabolic pathways mentioned in this work, with a focus on the compounds of major interest: verbenone, verbenol, isonovalal, and α-terpineol. Microorganisms belonging to the same genera tend to produce similar metabolites when the same carbon source is used, as is represented in Table 1. Furthermore, biotransformations using fungal strains are more abundant than those employing bacterial strains because the fungal metabolism is slower than that of bacteria, resulting in the production of desirable compounds with minimal structural modification and higher yields (Farooq et al. 2004). In this review, the information on biotransformation processes is subdivided according with the biocatalyst used: fungi, bacteria, and plant cell cultures.

Biotransformation using fungal strains

Main fungi: *Aspergillus* spp. and *Penicillium* spp.

Agrawal et al. (1999) reported the increase in the biotransformation of α-pinene to verbenol using *A. niger* and *Penicillium* spp. after treatment with ultraviolet irradiation, colchicine, or ethyl methanesulfonate (EMS). These treatments seek to improve the performance of the strain, as well as to increase the productivity and the concentrations of products (Agrawal et al. 1999). The best results were obtained when ultraviolet irradiation was used. The biotransformation efficiency increased 15- and 8-fold, respectively, reaching 4566 μg/100 and 3698 μg/100 mL for *A. niger* and *Penicillium* spp. For the other treatments, an increase of 2-fold was achieved for colchicine treatment in the case of *A. niger*, reaching a final concentration of 652 μg/100 mL of verbenol. An 8-fold increase was obtained with EMS (2500 μg/100 mL of verbenol). For the *Penicillium* spp. strain, the colchicine and EMS induction caused an increase of 1.5- and 2-fold, resulting in 720 μg/100 and 944 μg/100 mL, respectively. In this study, the verbenone production was also monitored; however, there was a remarkable decrease in the formation of this compound in all the treatments and for both strains when compared to the wild-type strain.

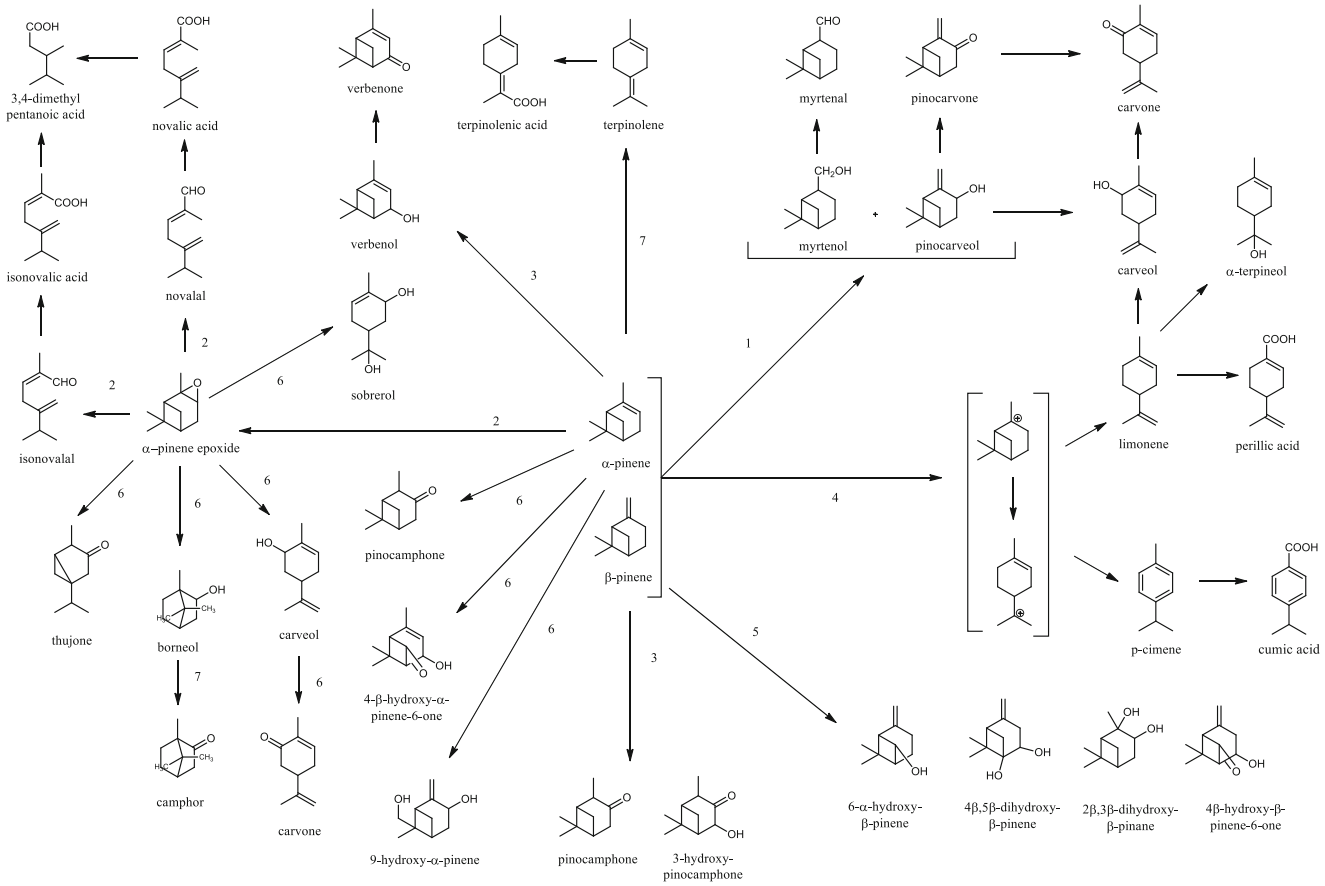

Fig. 2 Schematic representation of the main metabolic pathways of α- and β-pinene, discussed in this work: Savithiry et al. (1998) (1); Linares et al. (2009) (2); Van Dyk et al. (1998) (3); Yoo and Day (2002) (4); Farooq et al. (2002) (5); Schrader (2007)-adapted (6); Narushima et al. (1982) (7)

Prema and Bhattacharyya (1962) identified the optimal conditions for bioconversion of a mixture of isomers from α-pinene (70% *d*-α-pinene and 30% *l*-α-pinene) by *A. niger*. The highest concentration of *d*-verbenone, *d-cis*-verbenol, and *d-trans*-sobrerol were obtained from 0.6% (*v*/*v*) of substrate, after 8 h of reaction at 28.5 °C. The yields of the products were lower at higher substrate concentrations, longer reaction times, or higher temperatures.

The formation of verbenol and verbenone from α-pinene takes place in a two-step reaction: first hydroxylases act converting α-pinene to verbenol, and then dehydrogenases convert this compound to verbenone (Agrawal and Joseph 2000). Interestingly, *cis*-verbenol is the product resulting from metabolism by *A. niger* (Prema and Bhattacharyya 1962), whereas *trans*-verbenol is derived from auto-oxidation of α-pinene (Prema and Bhattacharyya 1962; Krings et al. 2009).

In a later study, Agrawal and Joseph (2000) reported the biotransformation of α-pinene to verbenone. They obtained a better conversion rate of substrate to product, approximately 16.5%, after a period of 6 h and pH 7, and the optimum substrate concentration was 0.2 mg/mL. In this study, the glucose content was observed to interfere in the conversion rate; better results were obtained when the glucose concentration in the culture media was 60 mg/mL (7.28 μg/mL of verbenone), whereas concentrations of the order of 40 and 30 mg/mL resulted in a conversion of 30 and 95% less efficient, respectively. Verbenone is a colorless liquid with an odor typical of mint and camphor. It is obtained commercially through the extraction of pine and eucalyptus with an average cost of US$3000/kg for natural verbenone (Agrawal and Joseph 2000; Burdock 2010). This product is mainly used in the food industry for the formulation of bakery products and sweets, but it can also be used as a starting material in the synthesis of various organic compounds, such as bicyclic lactones. These applications and the value of this compound make the biotechnological production of verbenone of great interest (Wender et al. 1995; Burdock 2010; Sarmiento et al. 2011).

Studies of the biotransformation of α- and β-pinene conducted by Brazilian groups (Rozenbaum et al. 2006; Rottava et al. 2010a, b) furnished interesting results using *A. niger* as the biocatalyst. In these cases, the main product obtained was α-terpineol. α-Terpineol is a very valuable compound because of its typical floral aroma, and it is found in a great variety of flowers, leaves, and herbs, although the chemical route is used

Table 1 Biotransformation products of α- and β-pinene

Biocatalyst	Substrate	Products	Reference
Fungi			
Penicillium sp.	α-Pinene	Verbenol and verbenone	Agrawal et al. (1999)
Aspergillus sp.	α-Pinene	Verbenol, verbenone, and sobrerol	Prema and Bhattacharyya (1962)
		Verbenol and verbenone	Agrawal and Joseph (2000); Rozenbaum et al. (2006)
	β–Pinene	α–Terpineol	Toniazzo et al. (2005); Rottava et al. (2010b); Rozenbaum et al. (2006)
Candida tropicallis	α-Pinene	α-Terpineol	Chatterjee and Bhattacharyya (1999)
Pleorotus sapidus	α-Pinene	Verbenol and verbenone	Krings et al. (2009)
Absidia corulea	α-Pinene	α-Terpineol and isoterpineol	Siddhardha et al. (2012)
Chrysosporium pannorum	α-Pinene	Verbenol and verbenone	Trytek et al. (2015)
Hormonema sp.	α-Pinene β-Pinene	Verbenol and verbenone Pinocamphone and 3-hidroxipinocamphone	Van Dyk et al. (1998)
Bacteria			
Pseudomonas rhodesiae	α-Pinene	Isonovalal	Fontanille et al. (2002); Bicas et al. (2008);
		Novalic acid	Linares et al. (2009)
Pseudomonas fluorescens	α-Pinene	Isonovalal	Bicas et al. (2008); Zorn et al. (2004); Boontawan and Stuckey (2006)
Mutant strain of *Pseudomonas fluorescens* NCIMB 11671	α-Pinene	Myrtenol and myrtenal	Colocousi et al. (1996)
Pseudomonas PIN	α-Pinene β-Pinene	Limonene, p-cimene, terpinolene, α-terpineol, and borneol	Yoo et al. (2001); Yoo and Day (2002)
Serratia marcescens	α-Pinene	Verbenol, verbenone, and sobrerol	Wright et al. (1986)
Bacillus pallidus BR425	α-Pinene	Carveol, carvone, pinocarvone, and pinocarveol	Savithiry et al. (1998)
Plant cell culture			
Picea abies	α-Pinene	Verbenol and verbenone	Lindmark-Henriksson et al. (2003); Vaněk et al. (2005)
	β-Pinene	Pinocarveol, myrtenol, α–terpineol, pinocamphone, and pinocarvone	Lindmark-Henriksson et al. (2004)

for commercial production (Toniazzo et al. 2005; Burdock 2010). Its annual consumption is around 20,300 lbs, having applications in condiment industry, bakery products, and sweets (Burdock 2010). As shown in Fig. 2., Route 4, α-terpineol is an oxidized product that can be obtained from both α- and β-pinene, as well as from limonene, another monoterpene of great interest found in many residues (Rozenbaum et al. 2006).

After a screening of 400 strains of microorganisms, three different strains of *A. niger* (ATCC 16404, ATCC 9642, and ATCC 1004) were shown to biotransform (−)-β-pinene into α-terpineol at 25 °C and 150 rpm for 6 days to furnish product concentrations varying from 0.172 to 2.856 g/L (Rottava et al. 2010b). Furthermore, after optimization experiments, the production of α-terpineol reached about 15.5 g/L using *A. niger* ATCC 16404 strain at 35 °C.

Toniazzo et al. (2005) studied the effect of cell induction with the monoterpene substrate (1% *v*/*v*) and the use of ethanol as co-solvent in the process of bioconversion of (−)-β-pinene to α-terpineol by *A. niger* ATCC 9642. The best results

were obtained with cells that were not precultivated with the substrate (non-inducted), in the presence of ethanol and with gradual addition of substrate. Accordingly, it was proposed that that the addition of substrate should be gradual to minimize the eventual toxicity and subsequent inhibition of biocatalyst cells, and thereby, facilitate the biotransformation of β-pinene (Toniazzo et al. 2005).

The potential biotransformation of α- and β-pinene by cultures of *A. niger* IOC-3913 was verified in four reaction systems: liquid media with growing cells, a precultivated strain, cell immobilized in synthetic polymer network, and solid media with substrate addition in the gas phase. For both substrates, the system with immobilized cells furnished the highest concentrations of products, being that the use of α-pinene as substrate lead to the production of verbenone, verbenol, and α-terpineol, whereas only α-terpineol was obtained from β-pinene (Rozenbaum et al. 2006).

The use of genetic engineering proved to be very effective in improving conversion rates of α-pinene to verbenol. A protoplast fusion of two strains of *A. niger* was performed. The first was a mutant strain with a conversion rate (α-pinene to verbenol) of 15.6%; the second was an α-pinene-resistant strain that presented a conversion rate of 26.4%. The resulting mutant strain obtained after protoplast fusion achieved a conversion rate of 48.6% and a productivity of 0.2 mg/mL after 6 h. These results are considerably higher than those obtained with a wild-type strain (conversion rate about 2%) (Vidya and Agrawal 2003).

Others

Candida tropicallis MTCC 230 was used in the biotransformation of α-pinene to (+)-α-terpineol at a concentration of 0.5 g/L, with continuous agitation for 96 h at 30 °C. This microorganism proved to be very efficient in the conversion of this substrate under these conditions, reaching a conversion rate of 77% and a production of 0.43 g/L (Chatterjee and Bhattacharyya 1999).

A comparative study of the auto-oxidation of α-pinene at 100 °C, and the biotransformation of this compound by strains of the *Pleurotus sapidus* basidiomycete suggested that the primary radicals formed in these processes are the same because the compounds formed in both processes are similar, with emphasis on the production of verbenol and verbenone. This study also shows that the use of concentrated or lyophilized mycelium was more efficient in the bioconversion than when submersed cultures were used (Krings et al. 2009).

The biotransformations of (−)-α-pinene and (+)-α-pinene to α-terpineol and isoterpineol were performed using cultures of *Absidia corulea* MTCC 1335 during 72 h with the addition of 60 mg of substrate. The best conversion rates were achieved when (+)-α-pinene was the substrate; the production of 34.116 mg of α-terpineol and 9.528 mg of isoterpineol was obtained, whereas the production of 28.146 mg of α-terpineol and 9.774 mg of isoterpineol was obtained from the enantiomer ((−)-α-pinene). Although this microorganism was more efficient in the biotransformation of one isomer, the results indicated that the enzymes involved in the processes are not enantioselective (Siddhardha et al. 2012).

The biotransformation of (−)-β-pinene by the pathogenic fungus *Botrytis cinerea* was described by Farooq et al. (2002). They obtained the following products and their yields after a period of 10 days: 6α-hydroxy-β-pinene (22%), (−)-4β, 5β-dihydroxy-β-pinene (10%), (−)-2β, 3β-dihydroxy-β-pinane (12%), and (−)-4β-hydroxy-β-pinen-6-one (9%) (Fig. 2, route 5) (Farooq et al. 2002).

The yeast *Hormonema* sp. UOFS Y-0067, isolated from pine tree samples, metabolized α-pinene to produce *trans*-verbenol (0.4 g/L) and verbenone (0.3 g/L) after a period of 72 h. Pinocamphone (0.1 g/L) and 3-hydroxy-pinocamphone (0.2 g/L), respectively, were obtained from β-pinene (Van Dyk et al. 1998).

Rottava et al. (2010a) isolated 405 microorganisms (mainly yeasts and filamentous fungi) from residues from the orange juice industry, soil from citric fruit plantations, leaves from citric trees, and eucalyptus trunks. Among all the microorganisms, only 31 were able to bioconvert α-pinene to verbenol, most of them having been isolated from residues from the orange juice industry. The best production (125.56 mg/L of verbenol) was achieved with a yeast strain.

In a study with endophytic microorganisms isolated from different fruits, two unidentified fungal strains were found to bioconvert (−)-α-pinene into verbenone. They produced between 0.101 and 0.133 g/L in mineral media with shaking at 150 rpm and 30 °C during 144 h (Abrahão 2014). Another study demonstrated the biotransformation of (−)-α-pinene into verbenone (0.066 g/L), *cis*-verbenol (0.030 g/L), and myrtenol (0.024 g/L) by an unidentified fungal strain in a process using mineral media and 0.5% of (−)-α-pinene as the carbon source, shaking at 150 rpm and 30 °C and during 96 h (Paulino 2014).

Recently, Trytek et al. (2015) demonstrated that the psychotropic *Chrysosporium pannorum* shows promise for the biotransformation of (1S)-(−)-α-pinene because this process can be performed at temperatures below 15 °C and, thus far, presents the best yield of verbenol and verbenone among the other microorganisms studied. The highest concentrations of verbenol, about 325.8 mg/L, were reached using 1.5% (*v*/v) of substrate and 72-h-old mycelium, whereas the best result for verbenone (about 203.4 mg/L) was obtained using 1% (*v*/*v*) of substrate and 48-h-old mycelium. The sequential addition of substrate proved to be a very efficient strategy to increase the yield because it was 3-fold higher than that obtained with a single addition of substrate after 72 h, reaching a concentration of 1.3 g/L, summing verbenol and verbenone (Trytek et al. 2015).

Biotransformation using bacteria

Pseudomonas sp.

Some bacteria of the *Pseudomonas* genus initiate the metabolism of α-pinene through the oxidation of the double bond at carbons 1–2, forming an epoxide. This reaction is catalyzed by α-pinene monooxygenase with subsequent opening of the cyclobutane ring to form acyclic aldehydes, as reported by Gibbon and Pirt (1971) and by Burfield et al. (1989), as is presented in Fig. 2, Route 2. This oxygenation leads to the formation of α-pinene oxide, a precursor of two compounds of economic interest: isonovalal and novalal (Fontanille et al. 2002). Griffiths et al. (1987) suggested that this same metabolic pathway is followed in the biotransformation process using *Nocardia* sp. strain P18.3 (Griffiths et al. 1987). Novalal and isonovalal are two compounds with potential applications in soap and detergent industries. Novalal has a characteristic lemon odor, whereas isonovalal presents a complex aroma, with citrus, woody, and spicy nuances (Burfield et al. 1989).

The biotransformation of (+)-α-pinene by *Pseudomonas* PX1 resulted in the formation of (−)-methyl-3,4-dimethylvalerate and *trans*-carveol, among other metabolites, after a period of 48 h. The substrate (0.33%; *v*/*v*) was added every 18 h of the process (Gibbon and Pirt 1971).

Tudroszen et al. (1977) cultivated *P. putida* PIN11 cells using 0.2% sodium acetate as the carbon source. Three aliquots of 1% α-pinene were added to the media every 24 h. The metabolites obtained from this process were 3,4-dimethylvaleric acid, 3-isopropylbut-3-enoic acid, and 2-methyl-5-isopropylhexa-2,5-dienoic acid. The same strain, mutated with N-methyl-N′-nitro-N-nitrosoguanidine (NTG), was converted to the metabolites 3,4-dimethylvaleric acid, 2,5,6-trimethylhept-3-enoic acid, and 3-isopropylbut-3-enoic acid (Tudroszen et al. 1977).

In an induction study, *P. rhodesiae* PF1 was precultivated with the addition of 40 g/L of α-pinene to increase the amount of specific enzymes that are possibly related to the process of conversion of α-pinene oxide to isonovalal. After this treatment, the cells were either frozen or used directly for the culture, which was conducted with the addition of α-pinene oxide in a biphasic system. The cultures with fresh cells reached a concentration of 10 g/L of isonovalal after 4 h, whereas 60 g/L of isonovalal was obtained with frozen cells after 2.5 h of the process (Fontanille et al. 2002). The same authors optimized the biotransformation conditions in the biphasic system using frozen cells permeabilized with organic solvents (toluene, chloroform, and diethyl ether), with detergent (Triton X-100) or by sonication, which resulted in better diffusion of the product and substrate through the cell membranes and increased the rate of formation of isonovalal to 16 g/L h (Fontanille and Larroche 2003).

Bicas et al. (2008) evaluated the ability of *P. rhodesiae* and *P. fluorescens* to metabolize α-pinene, β-pinene, α-pinene oxide, limonene, β-pinene oxide, and turpentine using fresh cell cultures and crude enzymatic extracts. The best results with both microorganisms were achieved using α-pinene as the substrate for cell growth and α-pinene oxide in the biotransformation step. In the process using *P. rhodesiae*, the products obtained were isonovalal, novalal, and novalic acid, whereas novalal and novalic acid were obtained using *P. fluorescens*. The same study suggested that the epoxidation of α-pinene is dependent on cofactors present only in intact cells, whereas the formation of aldehydes from α-pinene oxide is independent of these factors because only isonovalal was produced with the use of the same substrates, but with crude enzymatic extract (Bicas et al. 2008).

A study developed by Linares et al. (2008) demonstrated that cultures of *P. rhodesiae* CIP 107491 in a biphasic system produced about 12 g/L of acids from α-pinene oxide, of which 80% was constituted by novalic acid (Linares et al. 2008). Linares et al. (2009) explored the metabolic pathways involved in this biotransformation process to optimize the production of novalic acid in a stirred aerated tank bioreactor. The study demonstrated that there is a dependence of enzymatic cofactors for the isomerization of isonovalal into novalal; this can be presupposed by the fact that this isomerization only occurred in the presence of active biomass, which is able to synthesize and provide the necessary cofactors to the reaction, which does not occur in the use of crude enzymatic extract. It was also suggested that aldehyde dehydrogenase would be NAD^+ dependent, requiring, therefore, oxygen in the conversion of novalal to novalic acid. In this study, 16 g/L of novalic acid was obtained in about 8 h, with a yield of approximately 40% (Linares et al. 2009).

Strains of *P. fluorescens* NCIMB 11671, with mutations induced by NTG, were cultivated in the presence of 0.5% α-pinene as the only carbon source. Some of the mutant strains were able to grow only in the presence of α-pinene oxide, indicating that mutations affect the gene that codifies α-pinene monooxygenase, whereas others only grow in the presence of α-pinene. The strains without α-pinene monooxygenase activity produced myrtenol and myrtenal, among other compounds. However, the pathway for the production of these compounds was shown not to be viable because no carbon and energy for growth of microorganism was provided (Colocousi et al. 1996).

Cultures of *P. fluorescens* NCIMB 11671 cells converted about 56% of the substrate (1.92 mg of α-pinene oxide/mg cell every 15 min) to isonovalal after 3 h, resulting in the production of 16 g/L. The isomerization rate of isonovalal to novalal increased from 56 to 67% when the cell culture was coincubated with macroporous Lewatit OC 1064 polystyrene, where the system assists in maintaining a constant

concentration of substrate in the medium and possibly reducing inhibition by substrate (Zorn et al. 2004).

The biotransformation of α-pinene oxide to isonovalal by *P. fluosescens* NCIMB 11671 was evaluated in a membrane bioreactor for biotransformation (MBB) operating in continuous mode. A total of 108 g/L of isonovalal was obtained after 400 h of the process. The use of this reactor proved to be efficient for biotransformation of substrates with inhibitor potential because the cells exhibited a half-life of 2 months (Boontawan and Stuckey 2006).

P. maltophilia converted α-pinene (0.5% *v*/*v*) to limonene, borneol, camphor, perillic acid, and 2-(4-methyl-3-cyclohexenylidene) propionic acid. The study suggested that the formation of limonene and borneol was the result of the protonation of α-pinene by reaction with an acid catalyst, whereas perillic acid and 2-4-methyl-3-cyclohexenylidene) propionic acid would be products of limonene oxidation. The camphor obtained in this process would represent an intermediate in the borneol formation reactions (Narushima et al. 1982).

Yoo et al. (2001) and Yoo and Day (2002) evaluated the use α- or β-pinene (1% *v*/*v*) as the single source of carbon and energy by *Pseudomonas* sp. PIN and obtained conversion rates on order of 33.5 and 58.8%, respectively (Yoo et al. 2001; Yoo and Day 2002). The principal products found in the bioconversion of these compounds were limonene, *p*-cymene, α-terpinolene, α-terpineol, and endo-borneol in both cases. Interestingly, a higher amount of *p*-cymene was found than in previous reports, indicating that this strain has a pathway that favors the production of this compound. In disagreement with previous studies, in which concentrations of substrate above 1.5% were sufficient to inhibit cell growth, no significant growth inhibition was observed with substrate concentrations up to 10% *v*/*v*. The authors assumed that limonene formation is an intermediate isomerization step in the bioconversion process of both substrates.

Limonene, the principal product of this biotransformation route, as seen in Fig. 2, Route 4, has a typical lemon, sweet, orange, and citric aroma. This compound is widely used in the food industry; in chewing gum production; in alcoholic beverages; and in bakery products, candy, and jelly, among other products, with an average consumption about 133,700 lb per year (Burdock 2010). It can also be used as a solvent for resins, for the synthesis of other chemical compounds, and for applications in rubber, paint, and dispersing agents for oil (Maróstica and Pastore 2007b).

Other

The *Serratia marcescens* produced *trans*-verbenol, verbenone, and *trans*-sobrerol when incubated with α-pinene (0.4% *v*/v) as the only carbon source. The addition of glucose (10 g/L) and potassium nitrate (1 g/L) to the culture media induced the microorganism to follow another metabolic pathway involving the degradation of α-pinene to produce exclusively α-terpineol (Wright et al. 1986). These findings may indicate that the presence of the terpenes could induce the synthesis of specific enzymes and leading to the bioconversion by cellular detoxification process (Molina et al. 2015).

Bacillus pallidus BR425 converted α-pinene (15 mM) to carveol (42 μM/L), carvone (18 μM/L), pinocarvone (6.3 μM/L), and pinocarveol (5.4 μM/L) after a period of 48 h. The metabolite concentration suffered a substantial decrease when the incubation time exceeded 48 h (Savithiry et al. 1998). The production of carveol and carvone can occur from both α- and β-pinene, as demonstrated in Fig. 2, Route 1, via two different pathways: the first route involves the isomerization of this compound to limonene, followed by oxidation to carveol, and the second route is via the oxidation of the pinenes to pinocarveol or pinocarvone, followed by isomerization to carveol or carvone, respectively (Savithiry et al. 1998).

Among the products obtained in this biotransformation process, carvone stands out. The levorotatory form presents a typical of mint odor, and its *d*-isomer presents the odor of cumin (Bauer et al. 2001). This product is mainly used in alcoholic beverage industries, chilled dairy products, and sweets, with an average annual consumption of approximately 191,000 lbs (Burdock 2010). *l*-Carvone is commercially synthesized from limonene, whereas *d*-carvone is obtained from fractionated distillation of cumin oil (Bauer et al. 2001; Burdock 2010). Another important compound is carveol, which has a characteristic mint odor and is used in the production of jelly, soft sweets, and chilled dairy products. It is currently produced synthetically from dinitrobenzoate or from the oxidation of limonene and carvone (Burdock 2010).

In a recent study, Deepthi Priya et al. (2015a, b) evaluated the capacity of a new bacteria strain, recognized as *Gluconobacter japonicus* MTCC 12284, to biotransform α-(+)-pinene under the optimum conditions of pH 7 and 30 °C and obtained high-value products such as verbenone, verbenol, *trans*-sobrerol and α-terpineol (Deepthi Priya et al. 2015a, b). The microorganism presented a differential potential for the production of verbenone, reaching the maximum production after 7 days with a conversion rate of 95.6% and a yield of 0.21 mg/mL (Deepthi Priya et al. 2015b). Other pathways that lead to the formation of oxygenated derivatives form α-pinene, such as thujone, camphor from borneol oxidation, carvone from carveol oxidation, terpinolene, among others, as presented in Fig. 2, routes 6 and 7 (Narushima et al. 1982; Schrader 2007), have been described.

Biotransformation by plant cell culture

The biotransformation of α-pinene by cell cultures of *Picea abies* resulted in the formation of mainly *trans*-verbenol (35–

45%) and verbenone (20–25%) after a period of 4 days, when all the α-pinene had been consumed. When the experiment was performed in 8 days, an increase in the amount of verbenone (68%) was observed. *Trans*-pinocaveol, myrtenol, *cis*-verbenol, sobrerol, and α-terpineol were also detected but in smaller amounts. This work suggests that peroxidases are involved in the biotransformation process (Lindmark-Henriksson et al. 2003). In a later study, the same cell culture was used for the biotransformation of β-pinene to *trans*-pinocarveol (50%), myrtenol (28%), α-terpineol (13%), pinocamphone (5%), and pinocarvone (0.4%) as the principal products after a period of 15 days. However, unlike the first study, the products obtained from β-pinene biotransformation by *P. abies* are of little economic interest (Lindmark-Henriksson et al. 2004).

Vaněk et al. (2005) cultivated *P. abies* cells, free or immobilized in alginate, with the addition of 40 μL of isomers of α-pinene ((+), (−), or a racemic mixture) for a period of 1 to 28 days. The free cells metabolized α-pinene rapidly to form the products in different relative amounts than when the immobilized cells were used, while the formation of *trans*-verbenol in a ratio of 34% occurred after 1 h of fermentation with the use of free cells, the presence of this product in similar amounts (42%) was observed only after 24 h using immobilized cells. The main product formed by cells immobilized in alginate was *trans*-verbenol, which was later oxidated to verbenone (92% of the products after 14 days) (Vaněk et al. 2005). Products such as isopinocamphone (2–5%), 8–hydroxy–*p*–menth–1–en–6–one (1–12%), pinocamphone, and *p*-cymen-8-ol were detected in immobilized cells. An interesting observation in this study is that the free cells take about 8 days to produce *trans*-verbenol, whereas 14 days of reaction are required by immobilized cells.

Conclusion

Because of the wide range of products of commercial interest that can be produced from α- and β-pinene, studies on the biotransformation of these compounds have been stimulated for decades. From a technological point of view, the biotransformation of α- and β-pinene has already shown great advances in recent years, although there are still challenges to be overcome by the aroma and fragrance industries. Thus, various types of process optimization have been successfully implemented on the laboratorial scale, with the use of biphasic systems, to provide a better dissolution of substrate and increase the permeability of the cells to organic solvents to facilitate the diffusion of the product to the extracellular media. Some microorganisms have shown promise for the biotransformation of α- and β-pinene, such as bacteria of the *Pseudomonas* genus and fungi of the *Aspergillus* genus, leading to the production of compounds of high economic value with satisfactory yields. The discovery of new microorganisms, such as *C. pannorum*, which act under very mild conditions and may facilitate the work with volatile substrates, is noteworthy. Although this is a promising area, many challenges still need to be overcome, such as low solubility, volatility, and toxicity of the substrates as well as the low product concentrations, and thus, it is expected that the joint application of the selection of new microorganisms, process optimization techniques, and the possibility of scaling-up may facilitate the production of compounds labeled as natural from such versatile and cheap substrates as the pinenes.

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Compliance with ethical standards

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

Conflict of interest The authors declare that there is no conflict of interest.

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