2013 4th International Conference on Food Engineering and Biotechnology
IPCBEE vol.50 (2013) © (2013) IACSIT Press, Singapore
DOI: 10.7763/IPCBEE. 2013. V50. 13

Biotransformation of Monoterpenes by Endophytes Isolated From Brazilian Fruits

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Abstract. The term “endophytes” includes a suite of microorganisms that grow intra and/or intercelullarly in the tissues of higher plants without causing over symptoms on the plants in which they live. These microorganisms represent a potential source of novel natural products for medicinal, agricultural and industrial uses, such as antibiotics, anticancer agents, biological control agents, and other bioactive compounds. Despite the great potential of these microorganisms, their potential has not been investigated for the biotransformation of terpenes for the production of novel flavor compounds. Therefore, the aim of the present work was to isolate these microorganisms from Brazilian fruits and to investigate their biotechnological potential. Accordingly, it was proposed the biotransformation of the monoterpenes limonene, citronellol, α-pinene and β-pinene for the production of new flavor compounds. A total of 10 fungal strains were isolated from Cupuaçu (*Theobromagrandiflorum*), Curauá (*Ananaserectifolius*) and Jamelão (*Syzygiumcumini*) and identified in the present work. The preliminary results showed that the strains were capable of withstanding high concentrations of the terpenes tested (2-10%) and able to use the substrates as sole carbon source. The fungal endophyte LBCC1 bioconverted α-pinene into verbenol (85% similarity in MS results and confirmed with commercial standard), started after 48 hours of contact with the terpene. Quantification of verbenol showed a maximum production around 85 hours, reaching 40 $mg.L^{-1}$, and its production occurred based on the biochemical reaction of hydroxylation of α-pinene. Meanwhile, the biotransformation of limonene by LBCC1 and LBCC2 resulted in limonene-1,2-diol. Although this product was recurrent from two strains, this pathway is well known in the literature using fungi as biocatalysts in biotransformation process. When the substrate was β-pinene, the strains LBJM2 and LBCR1 produced myrthenol and α-terpineol, respectively. In the latter case, the production achieved 58 $mg.L^{-1}$ after 44 hours of fermentation. The biotransformation of citronellol resulted in rose oxide, a very interesting product for the flavor industry. The products achieved in this paper are of great industrial interest and the biotransformation of terpenes by fungal endophytes appeared as a promising alternative for commercial production of these bioflavors. Thus, this work demonstrates a partial use of these microorganisms in biotechnological processes and their potential as source of new flavor compounds from the biotransformation of monoterpenes.

Keywords: Limonene; Pinenes; Citronellol; Bioconversion; Flavor compounds.

1. Introduction

The term “endophytes” includes a suite of microorganisms that grow intra-and/or intercelullarly in the tissues of higher plants without causing over symptoms on the plants in which they live, and have proven to be rich sources of bioactive natural products [1], [2]. Mutualism interaction between endophytes and host plants may result in fitness benefits for both partners [3]. The endophytes may provide protection and survival conditions to their host plant by producing a plethora of substances which, once isolated and characterized, may also have potential for use in industry, agriculture, and medicine [4].

Approximately 300 000 plant species growing in unexplored area on the earth are host to one or more endophytes [5], and the presence of biodiverseendophytes in huge number plays an important role on ecosystems with greatest biodiversity, for instance, the tropical and temperate rainforests [4], which are extensively found in Brazil and possess almost 20% of its biotechnological source [6]. Considering that only a small amount of endophytes have been studied, recently, several research groups have been motivated to

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evaluate and elucidate the potential of these microorganisms applied on biotechnological processes focusing on the production of bioactive compounds.

Endophytes provide a broad variety of bioactive secondary metabolites with unique structure, including alkaloids, benzopyranones, chinones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones, xanthones, and others [2]. Such bioactive metabolites find wide-ranging application as agrochemicals, antibiotics, immunosuppressants, antiparasitics, antioxidants, and anticancer agents [4].

The biotransformation process provides a number of advantages over chemical synthesis. Therefore, biotransformation is a useful method for production of novel compounds; enhancement in the productivity of a desired compound; overcoming the problems associated with chemical analysis; leading to basic information to elucidate the biosynthetic pathway [7]. The biotransformation of terpenes, specially limonene and α-pinene ($C_{10}H_{16}$) has been extensively employed for the production of volatile compounds with great industrial interest, since it allows the production of enantiomerically pure flavors and fragrances under mild reaction conditions [8].

Despite the great potential of these microorganisms, their potential has not been investigated for the biotransformation of terpenes for the production of novel flavor compounds. Therefore, the aim of the present work was to isolate fungal endophytes from Brazilian fruits and to investigate their biotechnological potential. Accordingly, it was proposed the biotransformation of the monoterpenes limonene, citronellol, α-pinene and β-pinene for the production of new flavor compounds.

2. Material and Methods

2.1. Screening of fungal endophytes

The selected fruits Cupuaçu (*Theobromagrandiflorum*), Curauá (*Ananaserectifolius*) and Jamelão (*Syzygiumcumini*) were purchased at a local market. After proper sterilization of the surface, parts of the fruits (including the seeds, pulp and internal part of peel) were evenly spaced in Petri dishes containing potato dextrose agar (PDA) medium. The Petri dishes were incubated at 30 °C and monitored every day to check the growth of microorganism colonies from the fruit segments. After isolation, the fungal strains were cultivated and preserved by periodic replications (once a week) on Yeast-Malt agar (YM: 10 $g.L^{-1}$ glucose, 5 $g.L^{-1}$ peptone, 3 g.L-1 yeast extract, 3 g.L-1 malt extract, pH 6.7). The selected fruits Cupuaçu (*Theobromagrandiflorum*), Curauá (*Ananaserectifolius*) and Jamelão (*Syzygiumcumini*) were purchased at a local market.

2.2. Biotransformation process

A 72h culture grown on agar in a Petri dish was divided amongst 50 mL of YM medium and homogenized under sterile conditions using an Ultra-Turrax® T18 (Ika, Wilmington, NC, USA) until complete disruption of the solid matter. After 72 hours of incubation at 30 °C and 150 rpm, the cell mass was concentrated an placed in 50mL of mineral medium. Biotransformation was started by adding 3 $g.L^{-1}$ of α-pineneand incubated at 30 °C and 150 rpm. Samples were extracted with the same volume of ethyl acetate (with decane as internal standard) at 0, 24, 48, 72 and 96 hours for analysis by gas chromatography and mass spectrometry (GC-MS) for detection, identification and quantification of the products formed and substrate consumption.

3. Results and Discussion

A total of 10 fungal strains were isolated from Cupuaçu (*Theobromagrandiflorum*), Curauá (*Ananaserectifolius*) and Jamelão (*Syzygiumcumini*) and identified in the present work as presented in Table 1.

A: Terpene used was limonene and concentration was measured as +: 0,1-2%, ++: 2-5%, +++: 5-10%. B: Sole carbon source used was limonene and evaluation was +: 1 fold enhancement of dry mass concentration, ++: 2-3 1 fold enhancement of dry mass concentration, +++: 3 fold enhancement or more of dry mass concentration

Limonene was first used as substrate to assess the potential of the fungal strains and the preliminary results showed that the strains were capable of withstanding high concentrations of the thisterpene (2-10%) and to use limonene as sole carbon source. Results are presented in Table 1.

Meanwhile, the biotransformation of limonene by LBCC1 and LBCC2 resulted in limonene-1,2-diol. Although this product was recurrent from two strains, this pathway is well known in the literature using fungi as biocatalysts in biotransformation process.The bioconversion of *R*-(+)-limonene with the strain LBCR1 resulted on an interesting end product, a monoterpene alcohol labeled α-terpineol. Figure 1 comprises the bio-oxidation of limonene for theproduction of α-terpineol. This compound has a lilac odour and is one of the most commonly used fragrance compounds, representing an important commercial product that is typically applied in soaps, cosmetics and flavor preparations [9].

Table 1. Fungal strains, fruit source and results collected with limonene as substrate.

Name	**Fruit**	**Terpeneconcentration**[A]	**Sole Carbon Source** [B]	**Product obtained**	**Product concentration ($mg.L^{-1}$)**
LBCC1	Cupuaçu	++	+++	Limonene-1,2-diol	Trace amount
LBCC2	Cupuaçu	++	+++	Limonene-1,2-diol	10
LBCC3	Cupuaçu	+++	++	-	-
LBCC4	Cupuaçu	+	+	-	-
LBJM1	Jamelão	+	+	-	-
LBJM2	Jamelão	+	+	-	-
LBCR1	Curauá	+++	++	α-Terpineol	14
LBCR2	Curauá	+	++	-	-
LBCR3	Curauá	+	++	-	-
LBCR4	Curauá	++	+	-	-

All other microorganisms were capable of using limonene as sole of carbon source for growth, but as no accumulation of metabolites was observed, itis suggested the complete degradation of this substrate to CO_2. Even without positive results for the biotransformation, it emphasizes the potential its use in the degradation of limonene in bioremediation processes.

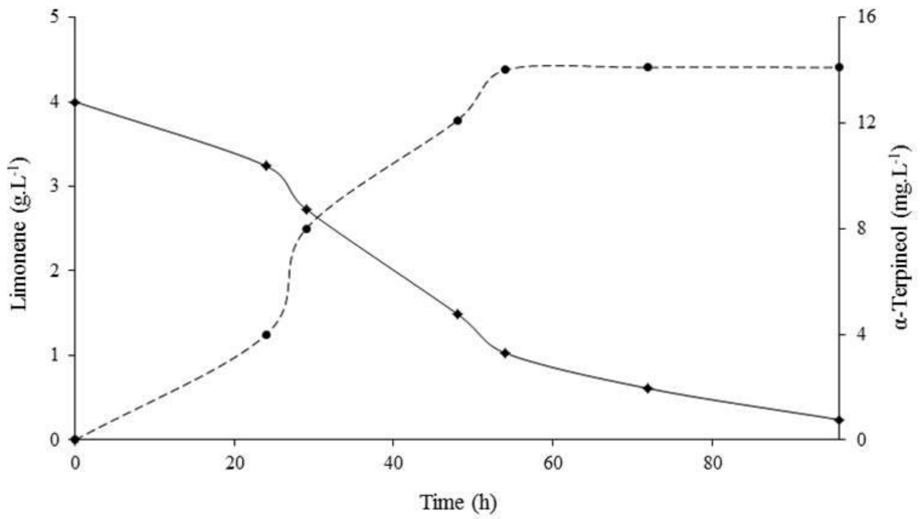

Fig. 1: – Bio-oxidation of limonene to α-terpineol by the fungal strain LBCR1. -♦- represents limonene consumption ($g.L^{-1}$) while --●-- represents α-terpineol production ($mg.L^{-1}$).

Fungal endophytes were also evaluated in biotransformation procedure using α-pinene ($C_{10}H_{16}$) as substrate, one of the most studied terpene in biotransformation process. This hydrophobic organic volatile compound is emitted from the forest products industry (e.g., wood products, pulp and paper industries).

Because of their economic advantage, pinenes represent an ideal substrate for biotechnological processes and have been extensively employed in microbial conversion experiments [10].

The fungal endophyte LBCC1 bioconverted α-pinene into verbenol (85% similarity in MS results and confirmed with commercial standard), started after 48 hours of contact with the terpene. Quantification of verbenol showed a maximum production around 85 hours, reaching 40 $mg.L^{-1}$.The biotransformation to verbenol occurred based on the biochemical reaction of hydroxylation of α-pinene (Figure 2), and this reaction was reported in some articles. Maróstica et al. [11] showed the biotransformation of α-pinene from the turpentine oil, by fungal strains, and a microextraction in solid phase was used for extraction of the aroma compounds. The production of 50 $mg.L^{-1}$ of verbenol and 70 $mg.L^{-1}$ of verbenone from α- and β-pinenes, respectively, was performed by Mucor sp. 2276. The production of verbenol/verbenone from α-pinene by Aspergillus sp. and Penicillium sp. strains was also reported by Agrawal et al. [12]. Further studies should be performed to optimize the process parameters to increase the production of this compound.

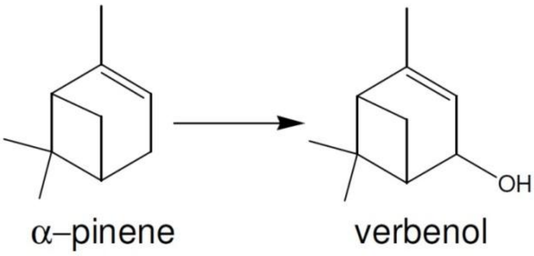

Fig. 2: Biochemical reaction of hydroxylation of α-pinene to verbenol.

When the substrate was β-pinene, the strains LBJM2 and LBCR1 produced myrthenol and α-terpineol, respectively. In the latter case, the production achieved 58 $mg.L^{-1}$ after 44 hours of fermentation.The biotransformation of citronellol resulted in traces of rose oxide, a flavour-impact component that occurs in traces in some essential plant oils, such asBulgarian rose oil, and it is considered one of the most important fragrance materials in creating rosy notes for perfumery and for the flavor industry. Despite its great interest, the concentration obtained in this process was found at low levels, hinderingthe biotechnological product forindustrial use or future processes optimization.

4. Conclusion

The products achieved in this paper are of great industrial interest and the biotransformation of terpenes by fungal endophytes appeared as a promising alternative for commercial production of these bioflavors. Thus, this work demonstrates a partial use of these microorganisms in biotechnological processes and their potential as source of new flavor compounds from the biotransformation of monoterpenes.

5. Acknowledgements

The authors acknowledge *Fundação de Amparo a Pesquisa do Estado de São Paulo* (Fapesp) and *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (Capes) for the financial support.

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