BIOTRANSFORMATION OF LIMONENE TO LIMONENE-1,2-DIOL BY TWO Colletotrichum SPECIES

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RESUMO – O objetivo do trabalho foi o estudo da biotransformação do limoneno por C. acutatum e C. nymphaeae. Inicialmente efetuou-se teste de resistência a terpenos e uso destes como única fonte de carbono pelos fungos. R-(+)-limoneno e terpeno cítrico foram usados como substrato para biocversão por biomassa crescida em meio com extrato de levedura e malte. As duas cepas foram resistentes a terpenos e usaram esses compostos como única fonte de carbono. Foram obtidas concentrações de limoneno-1,2-diol de 3,34 e 1,48 g.L⁻¹ para C. nymphaeae e C. acutatum, respectivamente, usando terpeno cítrico como substrato da biotransformação. O processo foi caracterizado como uma biotransformação aeróbica e dependente de cofator. As cepas apresentaram potencial para valorização de subprodutos agroindustriais. Este foi o primeiro relato de biotransformação de terpenos pelo gênero Colletotrichum. Estudos de caracterização do sistema enzimático, otimização de produção, recuperação e aplicação do produto obtido são encorajados.

ABSTRACT – The aim of this work was to study the biotransformation of limonene by C. acutatum and C. nymphaeae for the production of aroma and bioactive compounds. Initially we determined the resistance to terpenes and evaluated the use these substrates as the sole carbon source by such fungi. R-(+)-limonene and citrus terpene were used for bioconversion assays with biomass grown in yeast and malt broth. The two strains showed terpene resistance and used these compounds as sole carbon source. Concentrations of limonene-1,2-diol of 3.34 and 1.48 g.L⁻¹ were obtained for C. nymphaeae and C. acutatum, respectively, using citrus terpene as substrate. The process was characterized as aerobic and cofactor-dependent biotransformation. This was the first reported biotransformation of terpenes by Colletotrichum genus. Both strains showed potential for adding value to of agro-industrial by-products. Studies for the characterization of the enzyme system, production optimization, recovery and applications of the product obtained are encouraged.

PALAVRAS-CHAVE: Colletotrichum acutatum; Colletotrichum nymphaeae; 8-p-Menthene-1,2-diol; subprodutos; terpeno cítrico.

KEYWORDS: by-products; citrus terpene; Colletotrichum acutatum; Colletotrichum nymphaeae; 8-p-Menthene-1,2-diol.

1. INTRODUCTION

Flavors and fragrances have wide application in food, feed, cosmetics, chemical and pharmaceutical sectors. This substances represents a global market that moves billions of dollars and has estimated annual growth of 3.6% between 2015 and 2020 (Lucintel, 2015). The biotechnological production aroma compounds has emerged as an attractive alternative because it has the advantage, compared to chemical methods, to possess higher regio and enantio-selectivity, to present reduced or
no toxic residues and to result in products labeled as “natural” (Bicas et al., 2009). Therefore, the interest for the so-called “biotech” aroma is constantly increasing (UBIC, 2014).

Terpenes are substances synthesized by plants presenting roles in communication and defense against various stresses (Singh & Sharma, 2015). The monoterpene limonene is the major component of citrus oils. An estimated 31.2 million tons of citrus fruits are processed each year in the world, producing 15.6 million tons of wastes, which are rich source of limonene (Lin et al., 2013). Therefore, this compound is available in large amounts and low price. Although limonene has numerous applications, one of its most interesting destinations may be the production of value-added products, such as aroma compounds. Many of its oxygenated terpene derivatives, for instance, are used as fragrances and flavors (de Carvalho & da Fonseca, 2006).

Studies on the biotransformation of terpenes have been conducted using some phytopathogenic fungi because these micro-organisms are presumably adapted to these compounds (resistance and use as sole carbon source) (Miyazawa et al., 2002; Molina et al., 2015). Colletotrichum is a fungal genus with many species and may cause diseases in a wide range of plants (Cannon et al., 2012). Colletotrichum acutatum and C. nymphaeae species exhibit considerable genotypic and phenotypic diversity and are responsible for economically significant losses of temperate, subtropical and tropical crops, such as citrus and strawberry, for example (Damm et al., 2010; Damm et al., 2012).

Thus, considering the exposed, the main objective of this work was to study the potential of C. acutatum and C. nymphaeae to biotransform limonene and to characterize such process.

2. MATERIAL AND METHODS

2.1 Microorganism and chemicals

The strain C. acutatum TQ058A was gently supplied by Fundecitrus (www.fundecitrus.com.br, Araraquara, SP, Brazil). The strain C. nymphaeae CBMAI 0864 was a courtesy of the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI) of the Multidisciplinary Center of Chemical, Biological and Agricultural Research (CPQBA, Paulínia, SP, Brazil).

R(+)-limonene (purity ≥98%), (1S,2S,4R)-(+) -limonene-1,2-diol (97%≥%), (++)-cis-limonene-1,2-epoxide (97%≥%), (++)-trans-limonene-1,2-epoxide (99%≥%), (5)-(+-)perillyl alcohol (≥95%), (--)carveol (≥97%), (R)(--)-carvone (≥98% ), were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and solvents were of the best grade available. Was used in this study citrus terpene (produced from orange peel by the distillation process, 97% R(+)-limonene in GC, data not shown), gently supplied by Cocamar® (Paranavai, PR, Brazil).

2.2 Resistance to terpenes and its use as sole carbon source

A loopful of each strain was seeded in Petri dish containing solid mineral medium (MM) (in g.L⁻¹: NH₄Cl = 1; K₂HPO₄ = 0.5; MgSO₄.7H₂O = 0.02; agar = 17). The dishes were inverted and added inside the cover 100 µL of the tested terpene substrate (limonene, perillyl alcohol, carveol and carvone). The dishes were incubated (upside down) at 30 °C for 72 hours to monitor fungal growth (adapted from Cadwallader et al., 1989; Chang & Oriel, 1994).

2.3 Inoculum and biotransformation procedure

15 mL of saline (8.5 g.L⁻¹) was added to a Petri dish containing the strains previously grown in yeast and malt (YM) Agar (in g.L⁻¹: glucose = 10; peptone = 5; yeast extract = 3; malt extract = 3; agar = 20, pH ~ 6.7) at 30 °C for 72 hours. After manual scraping with Drigalski spatula, the resulting spore suspension was filtered through a double layer of cheesecloth and 5 mL of the filtered spore solution was inoculated into 125 mL conical flask with 50 mL YM Broth (consists of the same
materials as mentioned above without the agar). The flasks were incubated at 30 °C and 150 rpm for 72 hours for fungal growth.

The resulting biomass was recovered by vacuum filtration and, subsequently, it was resuspended in 50 mL phosphate buffer 20 μmol.L⁻¹ (pH 7) supplemented with 0.5% (v/v) terpenic substrate (R-(+)-limonene or citrus terpene) (adapted from Bicas et al., 2008). Two control experiments were done: an “abiotic biotransformation” with autoclaved biomass replacing the active biomass; and a fermentation process with no terpene addition. We also evaluated the biotransformation potential of other terpene substrates (for instance cis- and trans-(+)-limonene, 1,2-epoxide) to suggest by which pathway the microorganism is biotransforming limonene. In such cases, we used a smaller scale (10 mL of YM broth or phosphate buffer into 50 mL conical flask). Samples were collected every 24 hours to analyse the possible products formed.

For the bioconversion in anaerobic condition, the medium was flushed by bubbling N₂ for 5 minutes to remove oxygen dissolved in both the medium and headspace (Molina et al., 2015). This procedure was done at the beginning and after each sampling. All the other conditions were the same as described above. All biotransformations were performed in triplicate.

### 2.4 Identification and quantification of the volatile compounds

Samples were extracted (40 s in vortex) using the same volume of ethyl acetate. After phase separation, the organic fraction was dried over sodium sulphate and 1 μL of this phase was injected into a gas chromatograph with a flame ionization detector (GC-FID) HP-7890 (Agilent Technologies, Santa Clara, CA,) coupled to an HP-5 column (30 m length × 0.25 mm i.d. × 0.25 μm film thickness) operating at split mode (split ratio of 1:10). Helium was used as the carrier gas (1.0 mL min⁻¹), and the oven temperature was kept at 80 °C for 3 min, raised at 20 °C min⁻¹ until 200 °C and held for 4 min. The temperatures of the injector and detector were kept at 250 °C (Bicas et al., 2010). Substrates and products were quantified by a calibration curve using n-decane as an internal standard. All experiments were performed in triplicate.

The identification of volatile compounds was performed on a GC-MS system with a gas chromatograph HP-7890 coupled to a mass spectrometer HP-5975C (Agilent Technologies, Santa Clara, CA, USA). A column HP-5MS with 30 m length × 0.25 mm i.d. × 0.25 μm of film thickness was used to separate the volatile components. Helium was used as carrier gas at constant flow rate of 1.0 mL.min⁻¹. The programming of the gas chromatograph oven temperature was the same as mentioned above. The mass spectrometer transfer line was set at a temperature of 250 °C, impact energy of 70 +eV and a mass range 35-500 m/z. The identification of the compounds was made by comparing the spectra with NIST 2008 library over 90% similarity, and comparison with commercial standard.

### 3. RESULTS AND DISCUSSION

#### 3.1 Resistance to terpenes and its use as sole carbon source

It may be observed in Table 1 that the strains _C. acutatum_ and _C. nymphaeae_ showed growth in Petri dishes with solid MM and terpene as the sole source of carbon and energy. The absence of growth was only observed for _C. nymphaeae_ grown in carvone. The first step to select a potential terpene biotransforming microorganism is testing its resistance and its ability to use the substrate as the sole carbon source (Bicas et al., 2009). The use of terpenic substrate as the sole source of carbon and energy indicates the presence of a metabolic pathway for degradation of the substrate, which may possibly accumulate intermediates of interest (Bier et al., 2011). Therefore, both strains were considered as potential terpene biotransforming agents, eventually accumulating interesting compounds. For this reason they were tested in the biotransformation procedure.
Table 1 – Use of terpenes as the sole carbon and energy sources.

<table>
<thead>
<tr>
<th>Terpenic substrate</th>
<th>Growth after 72 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limonene</td>
<td>+</td>
</tr>
<tr>
<td>Alcohol perillyl</td>
<td>+</td>
</tr>
<tr>
<td>Carveol</td>
<td>+</td>
</tr>
<tr>
<td>Carvone</td>
<td>+</td>
</tr>
</tbody>
</table>

3.2 Biotransformation process

The two strains showed ability to biotransform limonene and to accumulate limonene-1,2-diol as main metabolite, using both R-(+)
limonene or citrus terpene as substrate (Table 2).

Table 2. Proportion (as percentage of GC peak area) of the main products formed after 144 hours of biotransformation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R-(+) Limmone</th>
<th>Citrus terpene</th>
<th>R-(+) Limmone</th>
<th>Citrus terpene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limonene-1,2-diol</td>
<td>93.9</td>
<td>97.2</td>
<td>96.6</td>
<td>96.9</td>
</tr>
<tr>
<td>Others</td>
<td>6.1</td>
<td>2.8</td>
<td>3.4</td>
<td>3.1</td>
</tr>
</tbody>
</table>

\[\text{Others}^\dagger \text{cis-(}+\text{-limonene-1,2-epoxide, trans-(}+\text{-limonene-1,2-epoxide and autoxidation products.}\]

Besides limonene-1,2-diol and limonene-1,2-epoxide, some trace compounds (“others” in Table 2) were also detected, but these were also observed in the “abiotic biotransformation”, indicating that such compounds are limonene autoxidation.

The biotransformation process using limonene-1,2-epoxide enantiomers as substrates showed that, in both cases, limonene-1,2-diol was produced for the two strains (data not shown). However, when (1S,2S,4R)-limonene-1,2-diol was the substrate, no compounds were found in the extract. This information supports the hypothesis that both strains present one of the six pathways for limonene degradation already described (Bicas et al., 2008): a pathway of converting limonene to limonene-1,2-diol via limonene-1,2-epoxide using the enzymes limonene 1,2-monooxygenase and limonene 1,2-epoxide hydrolase (Figure 1). This pathway was described by various authors as being a primary metabolism devoted to energy production (Bicas et al., 2008; de Carvalho et al., 2000; Molina et al., 2015; Schrader, 2007). In the case of Rhodococcus erythropolis, for example, such pathway starts with an attack at the 1,2 double bond of limonene by an FAD- and NADH-dependent monooxygenase. Subsequently, a very active limonene-1,2-epoxide hydrolase catalyses the hydrolysis of limonene-1,2-epoxide to limonene-1,2-diol (van der Werf et al., 1999). This fact may explain why no considerable accumulation of the epoxides were found (Table 2): in the case of Rhodococcus erythropolis, the limonene-1,2-epoxide hydrolase activity was 170 times greater than that limonene 1,2-monooxygenase activity in limonene degradation processes (van der Werf et al., 1999).

Figure 1. Proposed metabolic pathway of limonene degradation by C. acutatum and C. nymphaeae.
There were no products detected in the anaerobic biotransformation process. This is in accordance with previous reports on this pathway, which indicated the need for cofactors in the oxidation of limonene by limonene 1,2-monoxygenase.

Figure 2 shows the kinetics for the production of limonene-1,2-diol from limonene using two sources of substrates. It was evidenced that concentrations of 3.34 and 1.48 g.L\(^{-1}\) of limonene-1,2-diol were obtained for \(C.\) \(nymphaeae\) and \(C.\) \(acutatum\), respectively, using citrus terpene as substrate. When using standard substrate, the production was 2.34 and 1.41 g.L\(^{-1}\), respectively. Even under non-optimized conditions these concentrations are considerably high and near the maximal concentration recently described for the biotransformation of \(S\)-(–)-limonene to limonene-1,2-diol by \(Fusarium\) \(oxysporum\), i.e. of 3.7 g L\(^{-1}\) (Molina et al., 2015).

Figure 1. Biotransformation of \(R\)-(+)\-limonene (black symbols) or citrus terpene (white symbols) into limonene-1,2-diol by the fresh biomass of \(C.\) \(acutatum\) (triangles) and \(C.\) \(nymphaeae\) (circles) previously grown in YM broth.

Citrus terpene is a by-product originated from the pressing of orange waste (peel, bagasse and seed). It is mainly composed of \(R\)-(+)\-limonene. As a relatively inexpensive product, the use of biotechnological processes for the production of higher quality and value-added compounds is welcome, particularly in terms of marketing, technology and environmental aspects.

4. CONCLUSIONS

To the best of our knowledge, this was the first report of a limonene biotransformation by \(Colletotrichum\) genus. The concentrations of limonene-1,2-diol obtained, i.e. 3.34 and 1.48 g.L\(^{-1}\) for \(C.\) \(nymphaeae\) and \(C.\) \(acutatum\), respectively, were considered high and may be a good platform for the production of such compound. The biotransformation was characterized as aerobic and cofactor-dependent, requiring an aerated process with living cells. Thus, these strains have the potential for adding value to agro-industrial by-products. Studies for the characterization of the enzyme system, production optimization, recovery and application of the product are encouraged and some are already in progress in our group.

5. ACKNOWLEDGEMENTS

CNPq (process number 473981/2012-2).

6. REFERENCES


