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TRANSFORMATION OF *trans*-ANETHOLE USING THE PLANT PATHOGENIC FUNGUS *Colletotrichum acutatum* AS BIOCATALYST

TRANSFORMACIÓN DE *trans*-ANETOL USANDO EL HONGO FITOPATOGÉNICO *Colletotrichum acutatum* COMO BIOCATALIZADOR

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Abstract

Microbial transformation of propenylbenzenes may offer a cleaner and cheaper alternative to natural production of flavors and fragrances. In the present study, the biotransformation of *trans*-anethole using cells of a Colombian strain of the fungus *Colletotrichum acutatum* was investigated. Initially, fungitoxicity of this compound against *C. acutatum* was evaluated; *trans*-anethole displayed a relatively weak toxicity against the microorganism (<70%, at 200 μg/mL and after 48 hours) and apparently a detoxification mechanism was present. Then, the microorganism was incubated with the substrate at room conditions, using three different culture media (Czapek-Dox, Sabouraud and PDB). Results show that *trans*-anethole is mainly degraded through an epoxide-diol pathway (*trans*-anethole to anethole-epoxide, then to *syn*- and *anti*-anethole-diol, $p-$anisaldehyde, $p-$anisic acid and $p-$anisic alcohol). However, other minor metabolites [e.g. 3-(4-methoxyphenyl)-1-propanol, 1-(4-methoxyphenyl)-2-propanol, ethyl ester of anisic acid], possibly proceeding from other metabolic pathways were also found. Additionally, it was demonstrated that the concentration of metabolic products is dependent on culture medium used, being anethole-diol the major product obtained in all media used. Interestingly, some of the compounds generated in the biotransformation have been utilized as flavors and fragrances. Based on the identified metabolites, a possible metabolic pathway of the biotransformation of *trans*-anethole by *C. acutatum* was proposed.

Keywords: fungitoxicity, biotransformation, *C. acutatum*, metabolic pathway, culture media.

Resumen

La transformación microbiana de propenilbencenos puede ofrecer una alternativa más limpia y económica para la producción natural de aromas y fragancias. En el presente estudio se investigó la biotransformación de *trans*-anetol usando células de una cepa colombiana del hongo *Colletotrichum acutatum*. Inicialmente, se evaluó la toxicidad de este compuesto contra *C. acutatum*; *trans*-anetol exhibió una toxicidad relativamente baja contra el microorganismo (<70%, a 200 μg/mL y 48 horas) y aparentemente se presentó un mecanismo de desintoxicación. Luego, el microorganismo se incubó con el sustrato a condiciones ambientales, usando tres medios de cultivo (Czapek-Dox, Sabouraud y PDB). Los resultados muestran que *trans*-anetol es degradado principalmente a través de una ruta epóxido-diol (*trans*-anetol a anetol-epoxido, luego a *syn*- y *anti*-anetol-diol, $p-$anisaldehido, ácido $p-$anísico y alcohol $p-$anísico). Sin embargo, también se encontraron otros metabolitos minoritarios [por ejemplo, 3-(4-metoxifenil)-1-propanol, 1-(4-metoxifenil)-2-propanol, éster etílico del ácido anísico], posiblemente procedentes de otras rutas metabólicas. Adicionalmente, se demostró que la concentración de los productos metabólicos es dependiente del medio de cultivo usado, siendo anetol-diol el producto mayoritario obtenido en todos los medios. Interesantemente, algunos compuestos generados en la biotransformación se han utilizado como aromas y fragancias. Basados en los metabolitos identificados, se propuso una posible ruta metabólica para la biotransformación de *trans*-anetol por *C. acutatum*.

Palabras clave: fungitoxicidad, biotransformación, *C. acutatum*, ruta metabólica, medios de cultivo.

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1 Introduction

Flavors and fragrances are widely used in food, beverage, cosmetic, home and personal care, and in chemical and pharmaceutical industries (Palmerín-Carreño *et al*., 2014; Freire *et al*., 2005; Kashi *et al*., 2007). Nowadays, most of the flavor compounds on the world market are produced by chemical synthesis or by direct extraction from plants (Bicas *et al*., 2010). However, chemical synthesis results frequently in environmentally unfriendly production processes and the undesirable formation of by-products, thereby reducing process efficiency and increasing downstream cost (Longo and Sanromán, 2006; Wohlgemuth, 2010). Besides, natural flavor production by extraction from plants has some problems. First, the concentration of the desired compound is commonly low, making the extraction, isolation and formulation very expensive (Xu *et al*., 2007). Second, the level of flavor compounds in plants is influenced by different factors including weather, agronomic conditions, subspecies genotype, plant age and diseases (Longo and Sanromán, 2006; Bicas *et al*., 2010). Additionally, some flavors and fragrances are only found in exotic species. Owing to the demand for flavors and fragances, and the problems associated with organic synthesis and physical extraction from plants, the industry is actively pursuing new compounds and technologies for the production of natural flavors and fragrances (Shimoni *et al*., 2000).

In addition to the above, US (US Code, 1985) and European (The Council of the European Communities, 1988) legislations have defined that 'natural' flavor substances can only be prepared by appropriate physical, enzymatic or microbial processes, which involve precursors from natural origin. Recent market surveys have indicated that consumers prefer compounds labelled as 'natural', whereas other flavors that occur in nature but are produced by chemical methods, named 'nature-identical', are less appreciated (Serra *et al*., 2005). Due to the classification of 'natural' to biotechnology-derived compounds, research into production of flavors and fragrances through biotransformation systems has increased in recent years. The advantages of such approach are the relatively mild reaction conditions, the high substrate- or product-specificity, and its simple, cheap and benign methodologies that combine green chemistry with high efficiency (Kashi *et al*., 2007; Borges *et al*., 2009). Unfortunately, the productivity of such processes may be influenced by the toxicity of organic substrates and products, and the culture medium (Bertrand *et al*., 2013; Baeza-Jimenéz *et al*., 2014; León *et al*., 1998).

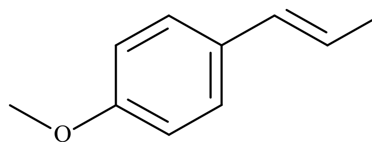

Fig. 1. Structure of *trans*-anethole (A)

In this sense, microbial transformation of propenylbenzenes has attracted considerable attention becoming one of the most important topics (Hua *et al*., 2007; Overhage *et al*., 2006; Yamada *et al*., 2007a, 2007b; Zhang *et al*., 2006). Propenylbenzenes, extracted from plant essential oils, are common aromatic compounds often employed as starting intermediates for the production of various flavors and fragrances. These compounds can be transformed by whole microorganisms or some enzymes to give natural flavors and fragrances which are products of high values (Koeduka *et al*., 2006; Xu *et al*., 2007). For example, the phenylpropanoids ferulic acid, eugenol, and isoeugenol have been used for preparing natural vanillin through biotechnological processes.

This study reports for the first time the ability of one strain of *C. acutatum*, which is a cosmopolitan filamentous phytopathogenic fungus that attack in harvest and postharvest, to biotransform *trans*-anethole [*trans*-1-(4-methoxyphenyl)-1-propene, (A), Figure 1] into value-added products. *trans*-Anethole is the major component on essential oils of Chinese star anise (*Illicium verum*), anise seed oil (*Pimpinella anisum*), and sweet fennel (*Foeniculum vulgare*). Additionally, the possible metabolic pathways of the biotransformation and the culture medium effect were discussed.

2 Materials and methods

2.1 Biological and chemical materials

The *C. acutatum* strain was provided by the Laboratory of Phytopathology (Universidad Nacional de Colombia-Medellin), was isolated from diseased *Solanum betaceum* cav. Sendt (tamarillo) fruits, and was characterized through morphological and molecular data by Dr. Afanador-Kafuri (Afanador-Kafuri *et al*., 2003). Compounds *trans*-anethole,

p−methoxycinnamic acid, *p*−anisaldehyde and *p*−methoxyphenylacetone were purchased from Alfa Aesar (Ward Hill, MA, USA). *p*−Anisic alcohol and *p*−anisic acid were prepared from anisaldehyde by reduction (with $NaBH_4$) and oxidation (CrO_3/H_2SO_4), respectively. Bacto agar, yeast extract, and peptone from casein (pancreatically digested) were obtained in that order from Becton, Dickinson and Co (Sparks, MD, USA), Oxoid Ltd (Basingstoke, UK), and Merck KGaA (Darmstadt, GER).

2.2 *Fungitoxicity bioassay*

The toxicity of (A) against *C. acutatum* was tested at different concentrations of the compound (i.e. 100, 125, 150, 175 and 200 μg/mL) by poisoned agar technique (Velasco *et al*., 2010). In Potato Dextrose Agar (PDA) medium at 50°C, was added enough (A) till achieve the desired concentrations, and then the mixture was poured immediately into 9-cm-diameter Petri dishes. Later, a mycelial plug (6 mm in diameter) cut from the growing edge of 3-day-old culture of the fungus was transferred to each plate. The PDA medium devoid of compound served as negative control. The cultures were incubated at room temperature and the diameter of the mycelial growth was measured each 24 h. The incubation was finished when the mycelial mass of control had almost filled the Petri dish (ca. 144 h). The results shown are the mean values ± SD of three replicates. The fungitoxicity of (A) expressed as percentage inhibition of radial growth was calculated by the formula:

$$Inhibition(\%) = \left[1 - \frac{\text{Radial growth of treatment (mm)}}{\text{Radial growth of control (mm)}}\right] \times 100 \quad (1)$$

2.3 *Preculture of C. acutatum*

The fungus was inoculated into six 1.0 L Erlenmeyer flasks, containing 0.5 L of Czapek-Dox liquid medium (Solution A: glucose 50.0 g/L, yeast extract 1.0 g/L; Solution B: K_2HPO_4 5.0 g/L, $NaNO_3$ 2.0 g/L, $MgSO_4.7H_2O$ 0.5 g/L, $FeSO_4.7H_2O$ 0.01 g/L). The flasks were kept at 120 rpm and room temperature for 168 h. The biomass was collected by filtration, washed with water and employed to inoculate a new medium supplemented with the substrate for the preparative biotransformation and time-course experiments.

2.4 *Preparative biotransformation*

C. acutatum cells were inoculated in six 1.0 L Erlenmeyer flask containing 0.5 L of broth and the substrate at 150 μg/mL dissolved in ethanol 96% (final concentration of 0.2%). Cultivation was performed at room temperature and 120 rpm during 360 h. This broth, was used to isolate the metabolic products after being discarded the mycelium by filtration. To verify the presence of similar compounds in the pathogen, a fungal culture without substrate was carried out.

2.5 *Isolation and identification of bioproducts*

The broth saturated with NaCl was refrigerated, filtered and extracted with CH_2Cl_2 (3 x 1.0 L). After that, the broth was acidified with HCl till reach pH 2, and extracted once more with CH_2Cl_2 (2 x 1.0 L). Both extracts were combined, dried over anhydrous Na_2SO_4 and concentrated in vacuum; the final extract was chromatographed on a silice gel column, and eluted with an *n*-hexane-EtOAc gradient system (100:0; 90:10; 80:20; 70:30; 60:40; 0:100, v/v). Six fractions were collected and gathered according to TLC profiles. Fractions eluted using *n*-hexane-EtOAc (80:20 and 70:30, v/v) were mixed and fractionated by size-exclusion column chromatography over Sephadex LH-20 (100 x 2 cm) employing the mixture *n*-hexane-CH_2Cl_2-MeOH (50:25:25 v/v) as eluent. The column chromatography afforded four compounds (B; 12 mg), (E; 4 mg), (F; 3 mg), and (H; 8 mg) which were identified by MS, ^{1}H and ^{13}C NMR analysis. Metabolites (E), (F), and (H) were also confirmed by comparison with authentic samples. In addition, identification of metabolic compounds (C), (D), (G), (I), (J) and (K) was based on interpretation of their mass spectra and by contrast with the NIST 2002 Mass Spectral Library.

2.6 *Time-course experiments and effect of culture medium*

Precultured *C. acutatum* was transferred into fifteen 250 mL Erlenmeyer flasks containing 125 mL of the Czapek-Dox medium and the substrate at 150 μg/mL, and kept under the same conditions as for preculture during 360 h. The broth from each flask was taken daily and then, it was supersaturated with NaCl, refrigerated, filtered and extracted threefold with CH_2Cl_2. The extracts were evaporated to dryness under reduced pressure in a rotary evaporator

at temperature below to 40°C. Then, extracts were redissolved in chloroform and analyzed by TLC and GC-MS. Furthermore, the ratios among the substrate and products were determined based on the GC peak area; the results were expressed as relative abundances. Similarly, time-course experiments using Sabouraud (peptone from casein, 10.0 g/L; anhydrous alpha-D(+)-glucose, 40.0 g/L) and PDB (potato, 200.0 g/L; anhydrous alpha-D(+)-glucose, 20.0 g/L) liquid media were developed, but the sampling were made every 48 hours beginning with the second day (Velasco *et al*., 2012).

2.7 Analytical methods

Thin layer chromatography (TLC) was made on Merck Kiesegel 60 F_{254} 0.25 mm thick. Mixtures of *n*-hexane:EtOAc were used as the mobile phase. Column chromatography (CC) was performed using silica gel 60 (0.040-0.063 mm; Merck) or Sephadex LH-20. GC-MS analysis was carried out using a Hewlett-Packard 6890 (Agilent Technologies) gas chromatograph coupled with a HP 5973 MSD (Mass Selective Detector - Quadrupole type), employing a HP−5 column (30 m x 0.25 mm i.d.; coating thickness 0.25 μm). The chromatographic conditions were: column temperature, 50-250°C at 10°C/min and keep it five minutes; injector temperature, 150°C; detector temperature, 280°C; carrier gas, N_2 at 1 mL/min. Relative composition of each constituent was established from the peaks average area obtained in GC. Identification of some metabolites was based on interpretation of their mass spectra and comparison with authentic compounds and by contrast with the NIST 2002 Mass Spectral Library. NMR spectra were measured on a Bruker AMX 300 NMR spectrometer (^{1}H NMR, 300.12 MHz; ^{13}C NMR, 75.42 MHz).

3 Results and discussion

3.1 Fungitoxicity bioassay

Enzyme synthesis and cofactor regeneration are related to fungal metabolism, which in turn may be affected by the toxicity of organic substrates and products (Shitu *et al*., 2009). Hence, a common limitation of the biotransformation processes is the inhibition or toxicity presented by the starting substrate and its products. To determine the concentration to use in the biotransformation, the toxicity (expressed as *in vitro* antifungal activity) of (A) against *C. acutatum* was previously evaluated. Overall, compound (A) displayed a moderate to weak toxicity against *C. acutatum*, which can be seen in Figure 2, where the higher inhibition occurred during the first 24 hours (more than 85%, at concentrations above to 150 μg/mL). However after this period, the inhibitory effect was strongly decreased at all the evaluated concentrations. Indeed, the growth inhibition percentage after 120 h was only 22% at 200 μg/mL (highest concentration tested). This fact suggests that the toxicity of (A) can efficiently be attenuated by the fungus, possibly through a detoxification mechanism. The compound (A) has demonstrated to be effective against insects and different microorganisms including bacteria, yeast and fungal strains (Chang *et al*., 2009; Mohammed, 2009; Huang *et al*., 2010; Kubo *et al*., 2008; De *et al*., 2001). Huang *et al*. (2010) have reported that (A) displays strong antifungal activity against *Alternaria solani*, *Botryodiplodia theobromae* and *Rhizoctonia solani*, with IC_{50} (50% inhibitory concentration) values of 110, 90 and 70 μg/mL, respectively. Likewise, (A) inhibited severely the spore germination of *Magnaporthe oryzae* (83.50% inhibition at 1.0 mg/mL). Similarly, (A) was active against *Bacillus cereus*, *Escherichia coli*, and *Klebsiella pneumoniae* (Mohammed, 2009) and exhibited synergistic effects on the antifungal activity of phytochemicals, including polygodial, (2E)-undecenal, and dodecanol against *Saccharomyces cerevisiae* and *Candida albicans* (Fujita *et al*., 2007). In fact, some authors have suggested that the use of (A) as antimicrobial agent may be exploitable to prevent the deterioration of fruits and vegetables by bacteria and fungi (Kizil *et al*., 2005).

In order to explore the biotechnological potential of *C. acutatum* to biotransform (A) into value-added products, the microorganism was incubated with the

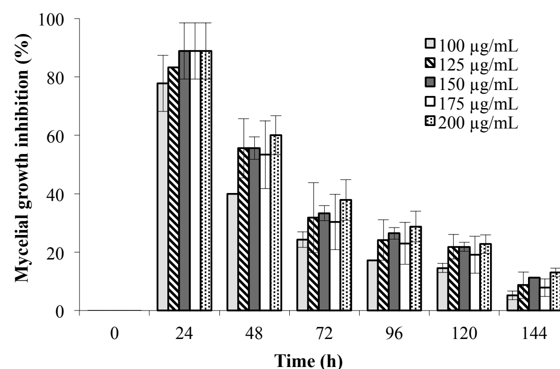

Fig. 2. Mycelial growth inhibition of *C. acutatum* by (A).

substrate at 150 μg/mL during 336 h. Such concentration was able to inhibit near to 85% of fungal growth at 24 h.

3.2 Isolation and identification of bioproducts

The isolation and identification of the main metabolic products was achieved from preparative incubation of (A) in Czapek-Dox with *C. acutatum*. During biotransformation, a pleasant and sweet odor was perceived, indicating the presence of flavor compounds. After 360 h incubation, the culture medium was filtered, extracted with CH_2Cl_2 and some compounds purified by means of CC, as was mentioned previously. A comparison by TLC and GC between the extract obtained from biotransformation and the control, showed that *C. acutatum* converted (A) into various metabolites. Initially, the structures of all metabolites were tentatively determined according to mass spectral data (Table 1), since some metabolites presented low concentration and were difficult to separate. However, Rf and Rt values of three of them were in accordance with authentic samples of *p*−anisaldehyde (E), *p*−anisic alcohol (F) and *p*−anisic acid (H). These compounds, along with (B) were also isolated and their structures totally confirmed by NMR.

According to the mass spectrum, metabolite (B) has a molecular formula $C_{10}H_{14}O_3$, $(M)^+ = 182$ amu. The molecular ion is 34 mass units higher than that of parent compound (A), indicating the addition of two hydroxyl groups to *trans*-anethole. Other spectral data confirmed the presence of two secondary hydroxyl groups (δ_H 3.87-4.10, and 4.33-4.64 ppm). It was concluded that metabolite (B) correspond to the mixture of diasteromers of 1-(4-methoxyphenyl)-1,2-propanediol (*trans*-anethole-diol). As can be seen in Figure 3, the ^{1}H NMR spectrum of (B) showed small peaks that can be attributed to its stereoisomer.

Table 1. GC retention time (Rt), EI mass spectral properties, and identification of major metabolic products detected in incubation of *C. acutatum* with *trans*-anethole (A).

Compound	*Rt* (min)	*m/z* of fragment ions [% relative intensity]	Identification
(B)	19.81 and 20.00	182 (M^+) [4], 138 [12], 137 (M^+ - CH_3CHO - H) [100], 135 [6], 109 [23], 94 [19], 77 [16], 66 [6], 65 [4].	Mixture of diasteromers of 1-(4-methoxyphenyl)-1,2-propanediol; anethole-1,2-diol * ‡
(C)	17.54	166 (M^+) [32], 148 (M^+-H_2O) [7], 147 [8], 122 [11], 121 (M^+ - H_2C=CH_2 - OH) [100], 105 [3], 91 [11].	3-(4-Methoxyphenyl)-1-propanol ‡,†
(D)	14.57	166 (M^+) [18], 122 [61], 121 (M^+ - CH_3CHO - H) [100], 107 [15], 91 [7], 78 [9], 77 [8], 65 [5], 45 [5].	1-(4-Methoxyphenyl)-2-propanol ‡
(E)	12.35	136 (M^+) [67], 135 (M^+-H) [100], 107 [17], 92 [18], 77 [35], 65 [12], 64 [11], 63 [14], 50 [11].	*p*-Anisaldehyde‡,†,•,*
(F)	12.86	138 (M^+) [100], 137 [59], 121 (M^+-OH)[45], 109 (M^+ - CO – H)[64], 107 [25], 105 [12], 94 [21], 79 [15], 77 [35], 39 [13].	*p*-Anisic alcohol‡,†,•,*
(G)	15.24 and 15.59	164 (M^+) [100], 149 [37], 137 [20], 133 [18], 131 [24], 121 [18], 103 [24], 91 [18], 77 [26], 55 [17].	Mixture of *syn*- and *anti*-anethole 1,2-epoxide‡,†
(H)	14.42	152 (M^+) [2], 136 [7], 135 (M^+ - OH)[100], 107 [5], 101 [2], 92 [6], 77 [8], 76 [2], 63 [2], 62 [3].	*p*-Anisic acid ‡,†,•,*
(I)	19.67	164 (M^+) [43], 122 [10], 121 (M^+ - CH_2CO – H)[100], 115 [8], 108 [37], 103 [12], 91 [19], 78 [8].	1-(4-Methoxyphenyl)-2-propanone‡,†
(J)	19.21	180 (M^+) [3], 137 [5], 136 [10], 135 (M^+ - H_2CO – CH_3)[100], 107 [6], 92 [8], 77 [13], 64 [4].	Ethyl ester of anisic acid‡
(K)	15.17	122 (M^+) [93], 121 (M^+-H) [100], 94 [6], 93 [41], 66 [6], 65 [42], 63 [7], 39 [30], 38 [6].	*p*-Hydroxybenzaldehyde‡,†

Identified by: *NMR; †mass spectrum identical with literature spectrum; ‡structure derived from the interpretation of the mass spectrum; •comparison with authentic standard or a sample synthesized from an authentic reference.

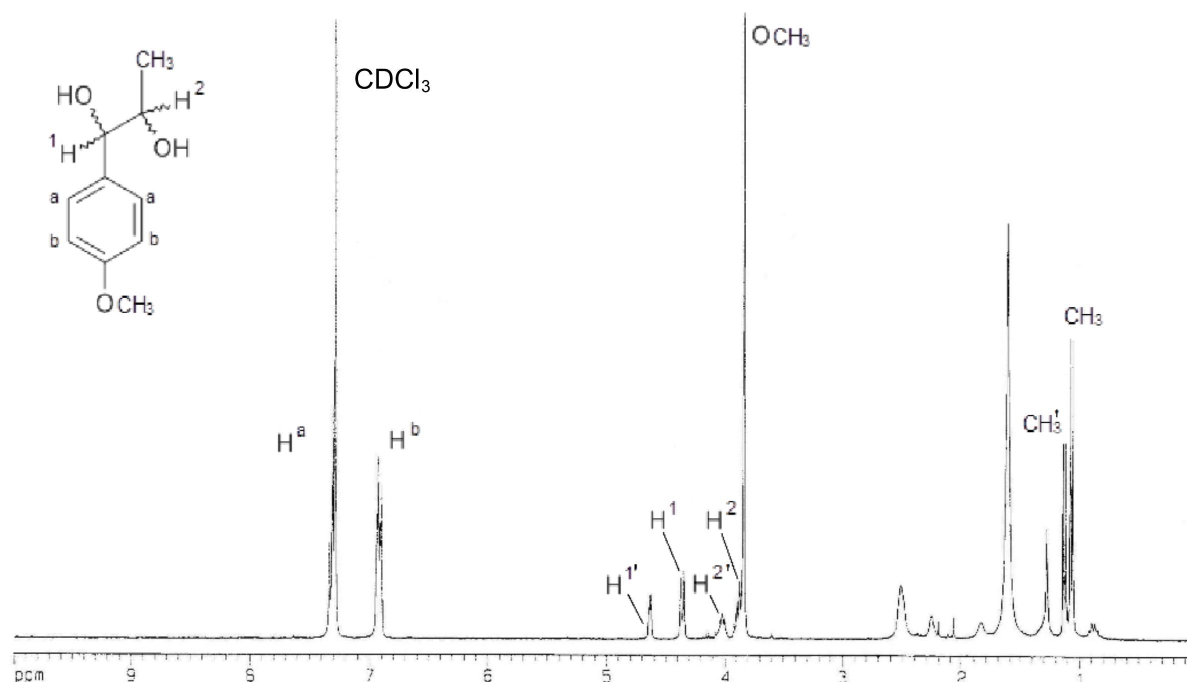

Fig. 3. ^{1}H NMR (300 MHz, $CDCl_3$) spectrum of (B) The symbols $H^{1'}$, $H^{2'}$, and CH'_3 denote the signals produced by another stereoisomer of the compound.

A similar finding was reported by Shimoni *et al*. (2002) from degradation of *trans*-anethole by *Arthrobacter aurescens*. The methyl signals of the minor stereoisomer (CH'_3) at δ 1.13 ppm and the major (CH_3) at δ 1.08 ppm are clearly separated; the ratio of integrals (CH'_3/CH_3) is about 3/5, indicating a small selectivity of biotransformation for one of the stereoisomers.

The molecular formula of metabolite (E) was $C_8H_8O_2$ according to its mass spectrum (M^+ = 136 amu). In addition, it was observed a prominent M-1 peak at m/z 135 (base peak) attributed to the loss of a radical hydrogen and formation of a $p-$methoxybenzoil cation. The mass spectrum of (E) was identical with that reported for $p-$anisaldehyde on the NIST 2002 Mass Spectral Library. The chemical structure of (E) was confirmed by co-elution, ^{1}H and ^{13}C NMR; δ_{1H}: 3.81 (3H, s, OMe), 6.93 (2H, d, J = 8.6, $H_{3,5}$), 7.86 (2H, d, J = 8.6, $H_{2,6}$), and 9.82 (1H, s, CHO). δ_{13C}: 55.9, 114.2, 123.0, 132.8, 163.0 and 195.0 ppm.

Metabolite (F) has a molecular formula of $C_8H_{10}O_2$ based on its mass spectrum, M^+ = 138 amu. Metabolite (F) has a fragment ion at m/z 121 attributed to the formation of a $p-$methoxybenzylic cation. From the ^{1}H spectral data; δ_{1H}: 2.04 (1H, s), 3.71 (3H, s), 4.63 (2H, s), 6.82 (2H, d, J = 8.2, $H_{3,5}$), 7.30 (2H, d, J = 8.2, $H_{2,6}$), (F) is elucidated to be anisic alcohol. The structure of (F) was confirmed by comparison with an authentic standard (TLC and GC co-elution).

Mass spectrum of metabolite (H) showed a molecular ion peak at m/z 152, in accordance with the formula $C_8H_8O_3$. Fragmentation pattern was consistent with the loss of hydroxyl radical, to give an abundant fragment ion (base peak) at m/z 135. The ^{1}H and ^{13}C NMR spectral data were undistinguishable from those reported for anisic acid. δ_{1H}: 3.94 (3H, s, OMe), 7.05 (2H, d, J = 8.8, $H_{3,5}$), and 8.11 (2H, d, J = 8.8, $H_{2,6}$). δ_{13C}: 55.5, 113.8, 121.7, 132.4, 164.1 and 171.4 ppm.

The characteristic fragment ion at m/z 121 (base peak), corresponding to the formation of $p-$methoxybenzylic cation, was used for identification of (C), (D) and (I). Whereas the base peak ion at m/z 135 (formation of $p-$methoxybenzoil cation), was found and used in (E), (H) and (J). The corresponding MS of (C), (D), (E), (H), (I), and (K) were indistinguishable from those reported in the NIST 2002 Mass Spectral Library. Additionally, mass spectral fragmentation patterns of (G) were in accordance to those reported by Kim *et al*. (1999) and Elgendy & Khayyat (2008).

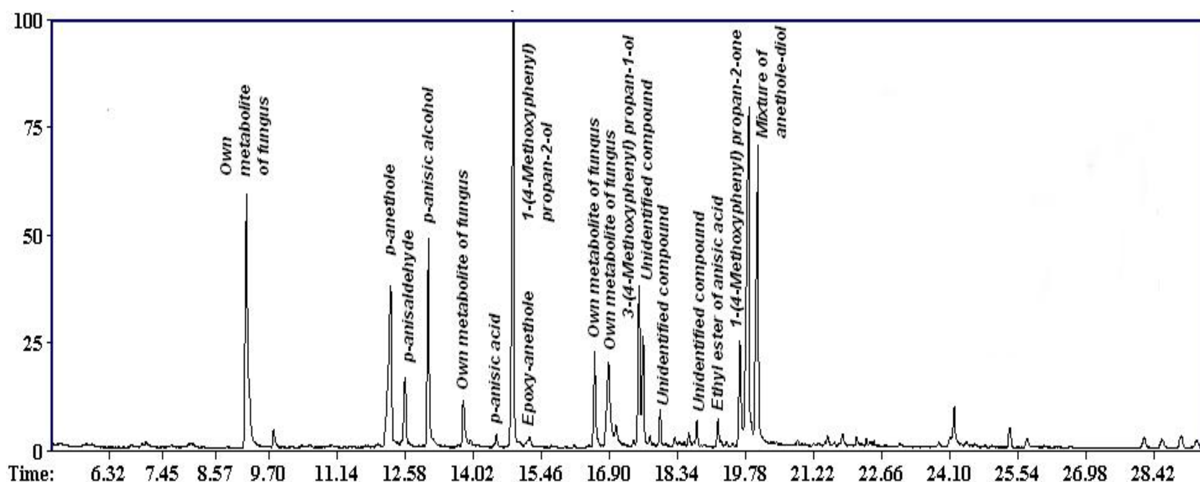

Fig. 4. Gas chromatogram of biotransformation of (A) by *C. acutatum* in Czapek-Dox medium after 48 h.

3.3 Time-course experiments and effect of culture medium

Daily samplings from the culture broth in Czapek-Dox supplemented with (A) were analyzed through TLC and GC (Velasco *et al*., 2012). In addition, all those metabolites were quantitatively measured through GC (Figure 4). Metabolite (H) was detected by GC-MS in all assays at trace level, due possibly to its relative nonvolatile nature. As it is shown in Figure 5, (A) was mainly transformed into (B) and (D). After 96 h about 90% of (A) was modified. Under the conditions used, the mixture of glycols (B) reached almost 40% of the products in 72 h, and then its concentration remained almost unchanged. In the same way, the secondary alcohol (D) reached about 36% at 312 h, and continued stable until the end of process.

Meanwhile, metabolites (C), (E) and (F) reached the maximum relative abundances at 144, 72 and 24 h, respectively. Metabolites (G), (H), (I), (J) and (K) did not reach an appreciable concentration during the evaluation (~2-4%). At traces level were also detected the metabolites 1-(4-methoxyphenyl)-1-propanol (1B), *Rt* (min): 15.18, MS m/z [%]: 166 (M^+) [13], 137 [100], 109 [28], and three unidentified compounds: two with indistinguishable mass spectra and with *Rt*'s (min): 17.62 and 17.98, MS m/z [%]: 210 (M^+) [2], 165 [100], 137 [67], 135 [15], 109 [20], and another one with *Rt* (min): 18.76, MS m/z [%]: 208 (M^+) [31], 207 [100], 135 [80], 108 [43]. Overall, chromatographic profiles showed a wide array of compounds that are own of the fungus or do not come from the biotransformation of *trans*-anethole.

A complementary study about the effect of culture medium on biotransformation of (A) was carried out. The microorganism was incubated with the compound (A) using PDB and Sabouraud media. Results of time-course experiments are shown in Figures 6 and 7. As can be seen (Figure 6), biotransformation employing PDB showed to be faster than the case of Czapek-Dox medium; substrate (A) was rapidly converted (about 95% after 24 h), mainly to metabolites (B), (D) and (J).

Overall, PDB medium demonstrated to be more suitable for transformation of (A) to (B); the mixture of stereoisomers of anethole-diol (B) reached about 93% at 48 h. However, the relative abundance was subsequently reduced to nearly 52% after 192 h, and afterwards, its concentration remained almost unchanged. During this process the metabolites (D) and (J) were also produced; they achieved maximum concentrations after 192 h (~35%) and 288 h (~18%), respectively. Under the conditions used, compound (J) which has not been seen before in Czapek-Dox medium was detected as one of the major products. Compounds (C), (E), and (I) were found in low abundances (< 5%) in PDB medium. Additionally, some metabolites at traces level were detected and tentatively elucidated according to MS, such as $p-$methoxypropiophenone (2B), *Rt* (min): 16.34, MS m/z [%]: 164 (M^+) [12], 135 [100], 77 [15]; 1-hydroxy-1-(4-methoxyphenyl)-2-propanone, *Rt* (min): 17.81, MS m/z [%]: 180 (M^+), 137 [100], 135 [20]; and 1-(4-methoxyphenyl)-1,2-propanedione, MS m/z [%]: 178 (M+), 135 [100]. Metabolites (F), (G), and (H) were not found under

these conditions. It seems remarkable that in PDB medium, the diol (B) reached a so different abundance (~95%) compared with Czapek-Dox medium (<40%).

Similarly, the biotransformation using Sabouraud medium demonstrated to be faster than on Czapek-Dox but slower than on PDB, as shown in Figure 7. In this culture medium, the substrate was almost completely biotransformed by *C. acutatum* within 48 h (>98%). Compound (A) was rapidly converted by fungus mainly to anethole-diol (B), which was the major metabolic product throughout the process, reaching the maximum concentration at 96 h (about 76%). Afterward, (B) decreased softly, indicating the formation of other metabolites, to achieve almost 61% at 192 h (and remained constant until 360 h). In fact, at the same time, (F) was increased to reach nearly 19% of relative abundance. Otherwise, metabolite (K) appeared within 24 h, and the maximum level was detected at 48 h (near 35% of the products). Next, relative abundance of metabolite (K) decreased rapidly. Under the conditions used, metabolites (C), (D), (E), (G), (H) and (I) were only found at low abundances (<15%). At traces level was detected 1-(4-methoxyphenyl)-1-propanol (1B).

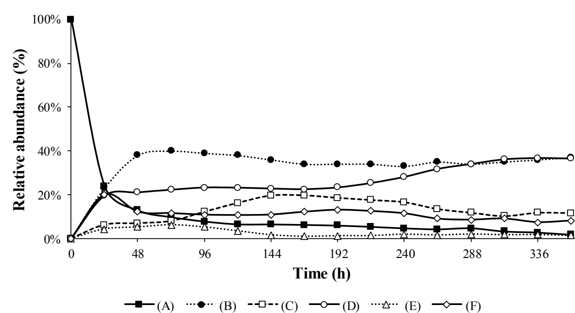

Fig. 5. Time-course for the biotransformation of (A) by *C. acutatum* in Czapek-Dox medium.

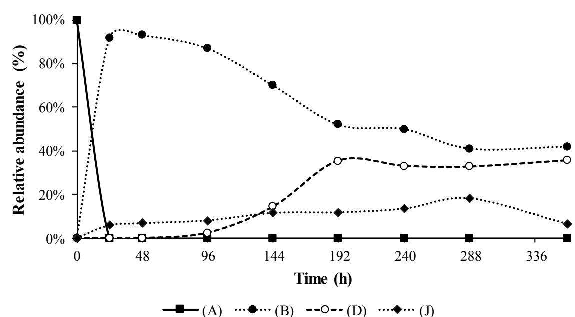

Fig. 6. Time-course for the biotransformation of (A) by *C. acutatum* in PDB medium.

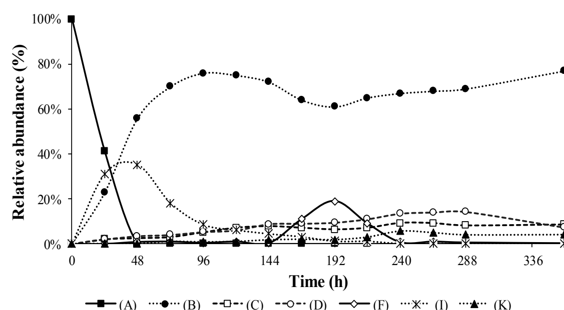

Fig. 7. Time-course for the biotransformation of (A) by *C. acutatum* in Sabouraud medium.

In general, in all media *C. acutatum* transformed the substrate (A) mainly to (B), the two isomers of 1-(4-methoxyphenyl)-1,2-propanediol, and about >99% of (A) was consumed after 24 and 48 h in PDB and Sabouraud media, respectively. The major metabolite, (B), made up about 93% of the products at 48 h in PDB medium. After reaching the highest abundance of (B) in Sabouraud and Czapek-Dox, it was remained almost constant until 360 h. In contrast, in PDB medium the abundance of (B) was rapidly decreased (52% at 192 h). Thus, PDB medium after 24 h of incubation can provide a simple and efficient way to obtain anethole-diol with high yield. However, further investigations to produce (B) in large quantities by optimizing the conditions of the biotransformation are needed. The metabolites (E), (F), and (H) were accumulated as minor products in the biotransformation of (A).

The variation in the specificity and production of metabolites in different media may be due to the enzymes presents in the microorganism are induced in different way, due to the suitability of each medium for the production of some specific metabolites. Thus, the Czapek-Dox medium, rich in minerals (Fe^{2+}, Mg^{2+}), could be providing metal ions needed to some catalytic processes (such as cofactors or Lewis acids), favoring certain stages involving redox reactions (enzymes involved in these reactions have cofactors which are present in the active site for electron transfer). In contrast, PDB medium, which is poor in minerals, showed the accumulation of few metabolites as compared to Czapek-dox and Sabouraud. Furthermore, the culture medium influences the physiological status of the fungus, which in turn, could induce differences in efficiency towards the formation of some products. These findings are in agreement with earlier results showing that the conversion of isoeugenol (a 1-phenylpropene analog of A) to vanillin and vanillic

acid might be individually influenced by inhibitors or metals that might selectively block or stimulate enzyme activities in their conversion (Seshadri *et al*., 2008). However, further investigations are needed to determine how the composition of culture medium affects the enzymatic behavior.

Although there are several studies about the *trans*-anethole metabolism in humans, rats, mice, and insects (Passreiter *et al*., 2004; Nakagawa and Suzuki, 2003; Bounds and Caldwell, 1996; Newberne *et al*., 1999), few studies have focused on the microbial biotransformation pathways, even more in plant pathogenic fungi. Initially, it was hypothesized that the metabolism of (A) by *C. acutatum* would be similar to other pathways reported for 1-phenylpropenes (Han *et al*., 2013a; Seshadri *et al*., 2008; Mishra *et al*., 2013; Priefert *et al*., 2001), which involves the oxidation of the side chain. Thus, based upon literature, three possible pathways were proposed for the conversion of isoeugenol by *Nocardia iowensis* DSM 45197 to vanillic acid and vanillin, although only one was substantially proven (Seshadri *et al*., 2008). In the same way, we proposed three probable paths for the transformation of (A) by *C. acutatum* (Figure 8). In *Path A*, compound (A) was transformed to *p*−anisaldehyde and then further converted to *p*−anisic acid or anisic alcohol. *Path B* shows hydration of the double bond of (A) to afford the 1-(4-methoxyphenyl)-1-propanol (1B), further oxidation to (2B), Baeyer-Villiger oxidation of (2B) to (J), and ester hydrolysis to (H). *Path C* shows hydroxylation of (A) to *p*−methoxycinnamyl alcohol, further oxidation to *p*−methoxycinnamyl aldehyde and then to *p*−methoxycinnamic acid. Finally, the *p*−methoxycinnamic acid suffers a β-oxidation to afford the anisic acid (H) (Figure 8). Nonetheless, the formation of cinnamic acid derivatives has been mainly reported from 2-propenylbenzenes, such as eugenol (Rabenhorst, 1996).

Taking in account the fact of metabolite (B) was isolated, the structure elucidated through MS and NMR, and quantified during the process, we can conclude that *C. acutatum* was able to oxidize (A) to (B) through an epoxide-diol pathway. *Pseudomonas putida* JYR-1, *Bacillus pumilus* and *B. subtilis* HS8 can also produce isoeugenol-epoxide and isoeugenol-diol as intermediates during the biotransformation of isoeugenol (Zhang *et al*., 2006; Hua *et al*., 2007). Also, two stereoisomeric epoxides, *syn*- and *anti*-anethole-epoxides, were identified as metabolic intermedies by Ryu *et al*. (2005). Furthermore, diol (B) was converted to anisaldehyde (E), and subsequently oxidized to anisic acid (probably catalyzed by a carboxylic acid reductase) or reduced to anisic alcohol (by an aldehyde oxidoreductase) (Li and Rosazza, 2000a,b; van den Ban *et al*., 1999). An identical metabolic pathway of (A) to anisic acid in two bacterial strains *Arthrobacter* sp. TA13 and *P. putida* JYR-1 has been suggested (Shimoni *et al*., 2002; Ryu *et al*., 2005). Thus, it shows an epoxidation of (A) to anethole-epoxide (G), epoxy hydrolysis to anethole-diol (B), and cleavage of anethole diol (B) to *p*−anisaldehyde (E), which is subsequently reduced to *p*−anisic alcohol (F) or oxidized to anisic acid (H) (Shimoni *et al*., 2003). The detection of (B), (E), (F), (G, as traces) and (H) indicates that *Path A* was operative in *C. acutatum*, and supports the hypothesis of Shimoni *et al*. (2003) that most propenylbenzenes (including *trans*-anethole, eugenol and isoeugenol) are degraded through an epoxy-diol pathway. The gene that codes for *trans*-anethole oxygenase (*tao*), which is able to catalyze the oxidative cleavage of the C-C carbono double bond on the 1-propenyl side chain of (A) to produce *p*−anisaldehyde was recently isolated from *P. putida* JYR-1 and expressed in *E. coli* (Han *et al*., 2012a,b). Additionally, a gene encoding *p*−anisaldehyde dehydrogenase (PAADH), which catalyzes the oxidation of *p*−anisaldehyde to *p*−anisic acid, was identified to be clustered with the *tao* gene (Han *et al*., 2013b).

Some authors indicate that the degradation pathway of *trans*-anethole by bacteria (*Arthrobacter* sp. TA13 and *P. putida* JYR-1) include the demethylation of *p*−anisic acid (H) to *p*−hydroxybenzoic acid (Shimoni *et al*., 2002; Ryu *et al*., 2005). Interestingly, the *p*−hydroxybenzoic acid was not detected in any culture media used. Nonetheless, when the biotransformation of (A) was carried out in Sabouraud medium, the metabolite (K) was found. The above could suggest a conversion of *p*−anysaldehyde (E) to *p*−hydroxybenzaldehyde (K) through an *O*-demethylase and is very likely to be further metabolized to *p*−hydroxybenzoic acid.

It is noteworthy that (D) was the second major metabolite found in biotransformation of (A) in Czapek-Dox and Sabouraud media. Metabolite (D) may be formed by the epoxidation of the propene side chain of *trans*-anethole (A), followed by the reductive ring opening of the epoxide (G). The alcohol (D) is subsequently oxidized, forming the ketone (I), likely by an alcohol dehydrogenase (*Path D*). This pathway has not been suggested before in the microbial metabolism of 1-propenylbenzenes. Remarkably, when *C. acutatum* was incubated with

the ketone (I), the metabolites (H) and (D) were found as major products (*Data not shown*). The transformation of (I) to (H) is quite rare, and to the best of our knowledge, it has not been reported before. The mechanism for this conversion is being actually investigated in our laboratory. Otherwise, our finding suggests reversibility in the conversion of (I) to (H).

Although some congeners of alcohol (C) have been previously reported in the microbial transformation of (A), the metabolite (C) itself results uncommon. According to information from literature (Seshadri *et al*., 2008; Gasson *et al*., 1998), (A) can be first hydroxylated to *p*−methoxycinnamyl alcohol, alcohol oxidation through *p*−methoxycinnamaldehyde to *p*−methoxycinnamic acid and β-oxidation of *p*−methoxyccinamic acid to anisic acid and/or anisaldehyde. In the present study, however, neither *p*−methoxycinnamyl alcohol, *p*−methoxycinnamaldehyde or *p*−methoxycinnamic acid were detected. The above is in agreement with the fact that while the 2-propene side chains are oxidized to 2-propenoic acids, the 1-propene side chains (i.e. A) are decarboxylated to form the corresponding substituted benzoic acid (Shimoni *et al*., 2003). Nevertheless, the formation of (C) appears to be resulting from the reduction of the C-C double bond in the *p*−methoxycinnamyl alcohol, possibly by an enoate reductase (Hall *et al*., 2006). The ability of *C. acutatum* to hydrogenate the C-C double bond of *trans*-cinnamaldehyde, *trans*-cinnamic acid and cinnamyl alcohol (analogue compounds of *p*−methoxycinnamyl alcohol) has been previously reported (Correa *et al*., 2009; Velasco *et al*., 2012).

The detection of metabolite (J) in high proportion, along with the low levels found of (1B) and (2B) suggest that either the *Path B* is functional, being the conversion of (A) → (1B) (oxidation) → (2B) (Baeyer-Villiger oxidation) fast processes. On the other hand, the production of (J) could be resulted from the esterification of acid (H), a capacity that has been reported for some microorganisms (Saerens *et al*., 2010). A similar metabolic pathway, (A) → (1B) → (2B) → (J) → (H) was proposed in the biotransformation of isoeugenol by *Nocardia* sp. (Seshadri *et al*., 2008).

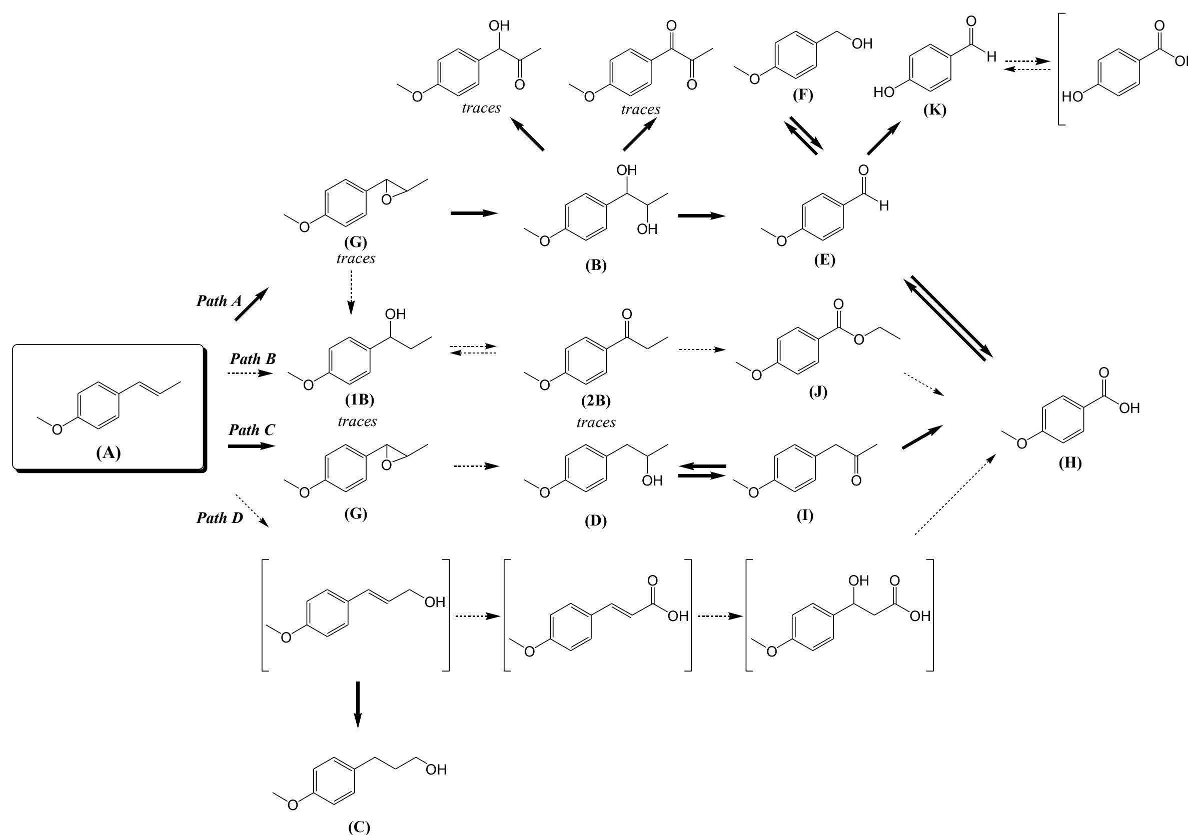

Fig. 8. Postulated metabolic pathways of (A) by *Colletotrichum acutatum*. Dashed arrows and brackets indicate the hypothetical steps and products in the metabolic pathways of *trans*-anethole, which have not been confirmed.

It is worth to point out that compounds (C), (D), (E) and (F), have an outstanding importance in the fragrance industry, and these are suitable substrates to several products of commercial importance (Bauer *et al*., 2001; Xu *et al*., 2007). Our study shed light on the paths by which (A) is oxidized by *C. acutatum* and emphasize the importance for further investigations to clarify the enzymes involved in the biotransformation.

Conclusions

The results reported here suggest that *C. acutatum* has the ability to oxidize the side chain of *trans*-anethole using several degradation pathways, being the epoxide-diol degradation the main route. Some products seem to come from metabolic pathways not previously reported. Additionally, the chemical profile of biotransformation products was depending to culture medium and incubation time. Overall, *C. acutatum* transformed *trans*-anethole mainly to the two isomers of anethole-1,2-diol in all media. Some compounds of great commercial importance in the flavor and fragrance industries such as *p*−anisaldehyde, *p*−anisic acid and *p*−anisic alcohol, 3-(4-methoxyphenyl)-1-propanol, 1-(4-methoxyphenyl)-2-propanol, 1-(4-methoxyphenyl)-2-propanone, and ethyl ester of anisic acid were also found in the biotransformation. Thus, *C. acutatum* has a considerable potential as biocatalyst for the preparative synthesis of these compounds. Nevertheless, further investigations to clarify the optimum conditions and the enzymes involved are needed.

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