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ORIGINAL ARTICLE

Structural characterization and metabolite profiling of the facultative marine fungus *Paecilomyces variotii*

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Abstract A new *Paecilomyces variotii* strain was isolated from the marine habitat. The fungal biomass necessary for the chemical study was produced with success, at a laboratory scale. A total of 28 structural groups were identified from volatile compounds that, in the main, are normal lipid compounds involved in the fatty acid pathway, fragments from their catabolism, terpenoids, and a metabolite from the shikimic acid route. In addition, two non-volatile compounds, triolein and ergosterol peroxide, were isolated and identified by spectroscopy. This is the first report to describe these compounds for the species *P. variotii*, suggesting its potential use as a natural source to produce nutraceuticals and functional foods.

Keywords *Paecilomyces variotii* · Facultative marine fungus · Lipid · Gas chromatography–mass spectrometry · NMR spectroscopy · Nutraceutical

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Introduction

The filamentous fungus *Paecilomyces variotii* is well-known for its biotechnological applications and for its ability to produce enzymes and new compounds. Its metabolic potential notably includes the production of a classical single cell protein, the Pekilo® biomass, manufactured by a continuous fermentation process, which has been used in the animal feed industry (Smith 2004).

The tannase obtained from this fungus has also been used in the enzymatic hydrolysis of tea extracts from *Camelia sinensis* and Yerba Mate *Ilex paraguariensis* (Battestin et al. 2008). The biotransformation of these phenolic acids allows for enhancement of the anti-radical properties of the tea extracts and is thought to contribute to the health benefits of the beverage (Macedo et al. 2011; Sachan et al. 2006).

The production of renewable fuels enables a reduced global dependence on fossil fuels, offering new opportunities for rural development. In this sense, the enzymes produced by fungal fermentation allow the use of agricultural biomass and waste to produce bio-fuel (Nigam and Singh 2011; Pandey et al. 2000). Through their ligninolytic enzymes, *Paecilomyces* species are able to deploy a large number of complex growth substances such as cellulose, and to convert them into value-added products such as bio-ethanol (Basso et al. 2010; Fu Wu 1985). Lipolytic enzymes can also catalyse the hydrolysis of several oils and fats obtained from these renewable sources, transforming them

into bio-diesel fuel (Jaeger and Eggert 2002; Vieira et al. 2006).

These advantages aside, *P. variotii* has also been employed in the reduction of cyclic terpenoids and aromatic aldehydes (Deodhar et al. 2002), as well as in the treatment and detoxification of residual waters and agricultural effluents (Vanhulle et al. 2003). In relation to their agriculture applications, it has been reported that the micromycete is particularly effective in combating the tomato disease produced by another fungus, *Fusarium* sp. (Wahid et al. 2001).

New and interesting substances, such as the herbicide cornexistin (Takahashi et al. 1994), the sphingofungins E and F (Horn et al. 1992), eicosenoic acids with antimycotic activity (Horn et al. 1993), and an antibiotic of mixed biogenesis, ascofuranone (Terekhova et al. 1997), have been isolated from the secondary metabolism of this fungus. Finally, its lipid composition and hydro-soluble polysaccharide structure have also been detected using GC, GC-MS and NMR spectroscopy (Dechkan-Khodzhaeva et al. 1997; Sunesson et al. 1995).

The present work reports the study of the metabolites biosynthesised by the fungus *P. variotii* as isolated from the marine biota in the Canary Islands. Detailed screening of the lipo-soluble and hydro-soluble fractions was carried out, and volatile and non-volatile compounds were identified using GC-MS and NMR spectroscopy. It is expected that intra-specific information with respect to the fungus' chemical diversity may offer crucial pointers towards a better understanding of the potential applications of this useful organism.

Materials and methods

Isolation and identification of the fungus

The fungus was obtained from samples of seawater collected in the intertidal zone of the "La Laja" beach, Gran Canaria, the Canary Islands, Spain. The isolation process and strain purification was carried out in Petri dishes on a modified KMV solid medium made from 1 g yeast extract, 1 g hydrolysed gelatine, 1 g peptone, 5 g glucose and 12 g bacteriological agar in 1 litre filtered seawater (35 ppt salinity). The fungus was identified by CABI Bioscience (Egham, Surrey, UK) as *Paecilomyces variotii* and a voucher specimen was preserved at the laboratory for future reference.

Production of mycelial biomass

Fungal biomass production was carried out in a static culture system, in a plastic box (83×46×18 cm) previously washed with detergent, sterilised with sodium hypochloride and steamed for 5 min. After the steam had condensed, the water was drained out of the boxes and the previously inoculated culture broth was introduced. The culture medium used for the fungal production was KMV modified broth made from 1 g yeast extract, 1 g hydrolysed gelatine, 1 g peptone, and 5 g glucose in 1 litre filtered seawater (35 ppt salinity) that was sterilised by autoclaving (20 min) and distributed into several boxes (2 L/ unit). After 10 days of incubation at 22–25°C, the supernatant mycelium was separated by filtration, and dried by IR radiation.

Apparatus and general procedures

Normal-phase chromatography was carried out on silica gel (Scharlau) with a 0.06–0.2 mm particle size for the adsorbent and 0.04–0.06 mm for the stationary phase. Chromatography was performed either at medium pressure (Büchi Chromatography System, http://www.buchi.com) or a low pressure with a Fluid Metering motor connected in series with an Ace Glass column (http://www.aceglass.com). Reverse-phase chromatography was carried out on a LiChroprep RP-18 (40–63 μm particle size, Merck, Darmstadt, Germany) column connected to a low-pressure chromatography system based also on a Fluid Metering (http://www.fmipump.com) apparatus. Size exclusion chromatography was carried out on lipophilic Sephadex® LH-20 (Sigma, St. Louis, MO). The column was eluted first with anhydrous methanol (2 h) and then with a mixture of CH_2Cl_2/CH_3OH (50:50, 2 h). The extracts were applied on the top of the column and eluted with CH_2Cl_2/CH_3OH (50:50) at a rate of 1.0 mL min^{-1}. Normal-phase TLC was performed on silica gel plates (0.25 mm diameter, Tracer Analitica, http://www.teknokroma.es) using a combination of *n*-hexane, ethyl acetate, chloroform and methanol as an eluent, in the proportions detailed for each case. Reverse-phase TLC was carried out on RP-18 F_{254} plates (0.25 mm, Merck) with the use of $CH_3CN/CH_3OH/H_2O$ (80:18:2) as a mobile phase. In all cases, the spots were revealed by spraying with oleum (sulphuric acid, 4%+acetic acid, 80%+water, 16%) and heating at 120°C for 20 min. Normal-phase semi-preparative HPLC was performed using an Alltech Econosphere C18 column (10 μm particle size, 250×4.6 mm, 100 Å pore size) and reverse-phase semi-preparative HPLC on a Waters ODS column (10 μm particle size, 250×4.6 mm, 100 Å pore size). Both of these processes were carried out on a semi-preparative HPLC apparatus coupled to a Spectra-physics P100 isocratic pump and used in line with a Hewlett Packard 1050 UV–vis variable wavelength detector, working at room temperature (26°C). Analytical chromatography was performed using a Shimadzu HPLC system with a LC-9A pump connected in line with a UV SPD-6AV detector (254 nm). The conditions used for the normal-phase column were combinations of n-hexane and ethyl acetate as eluent and, in the case of the size exclusion chromatography column (Shodex OH Pak SB 806 HQ), a

Table 1 Volatile organic compounds identified in the mycelium of *Paecilomyces variotii* by GC-MS

No	Retention time (min; mean±SD)	Compound (structure[a])
1	12.191	2,6-Octadienal, 3,7-dimethyl-, (E)- (**22**)
2	12.192	Hexadecanoic acid, 2-hydroxy-1, 3-propanodiol ester (**15**)
3	12.236±0.006	Tridecanoic acid (**4**; n=11)
4	12.369	2-Undecanone (**21**)
5	12.438	1-Tetradecene (**2**; n=11)
6	13.262	Tetradecane (**1**; n=11)
7	13.676	Nonanoic acid, 9-oxo-, methyl ester (**20**)
8	13.768	Decyl oleate (**17**; n=0)
9	14.001	Tetradecane, 2,6,10-Trimethyl- (**23**)
10	14.280±0.188	Pentadecane (**1**; n=12)
11	14.670±0.207	1-Tridecanol (**3**; n=11)
12	14.774±0.001	Tetradecanoic acid (**4**; n=12)
13	14.876	1-Hexadecene (**2**; n=13)
14	15.888	1-Pentadecanol (**3**; n=13)
15	16.249±0.024	n-Hexadecanoic acid (**4**; n=14)
16	16.339±0.014	1-Hexadecanol (**3**; n=14)
17	16.912	Heptadecanoic acid (**4**; n=15)
18	17.144	11-Hexadecenoic acid, methyl ester (**8**; n=3, m=9)
19	17.114	Docosanoic acid, 1,2,3-propaneTriyl ester (**13**)
20	17.261	Pentadecanoic acid, 14-methyl-, methyl ester (**11**)
21	17.298±0.006	Hexadecanoic acid, methyl ester (**5**; n=14)
22	17,583	Octadecanoic acid (**4**, n=16)
23	17.604	9-Hexadecenoic acid, methyl ester, (Z)- (**8**; n=5, m=7)
24	17.614±0.019	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester (**14**)
25	17.719	Hexadecanoic acid (palmitic acid), ethyl ester (**6**)
26	17.722	Nonadecanoic acid (**4**; n=17)
27	17.887	Hexadecanoic acid, octadecyl ester (**18**)
28	18.371±0.004	9-Octadecenamide (**9**)
29	18.420	Oleic acic, eicosyl ester (**17**, n=10)
30	18.516	9-Hexadecenoic acid, 9-octadecenyl ester, (Z,Z)- (**16**)
31	18.565±0.007	Octadecanoic acid, methyl ester (**5**; n=16)
32	18.596±0.221	9,12-Octadecadienoic acid (linoleic acid), ethyl ester, (Z,Z)- (**10**)
33	18.730	Octadec-9-enoic acid, (Z)- (oleic acid) (**7**; n=5, m=7)
34	18.817	9,12-Octadecadienoic acid (linoleic acid), 2-hydroxy-1-(hydroxymethyl) ethyl ester, (Z,Z)- (**19**)
35	19.800±0.024	13-Docosenoic acid (erucic acid) (**7**; n=7, m=11)
36	19.818	9-Octadecenoic acid, tetradecyl ester, (Z)- (**17**, n=4)

[a] "Structure" refers to the compound number in the figures

mixture of water and 0.05% sodium azide was used as an eluent. An eluent flow rate of 1.0 mL min^{-1} was used in all the analysis. Infra-red spectra were recorded on a Shimadzu FTIR-8400S spectrophotometer with chloroform (Merck) as a solvent for spectroscopy. The samples were sandwiched between two sodium chloride plates and the spectrum was calibrated against the 1,603 cm^{-1} band of polystyrene. ^{1}H- and ^{13}C-NMR spectra with two-dimensional experiments recorded at 250 or 300 MHz on AC or AMX Bruker apparatus, respectively. A Varian UNITY INOVA 400 MHz NMR spectrometer was used for high resolution analysis. Tetramethylsilane was used as an internal standard for ^{1}H and deuterated chloroform (δ 77.00) or deuterated methanol (δ 49.00) for the calibration of the carbon-13 NMR spectra. Electrospray ionisation mass spectrometry was performed either at low or high resolution with a common electron impact mass spectrometer (IE) or by fast atom bombardment (FAB). Positive-mode was carried out on a FAB-MS at 70 eV with a FISONS VG Micromass Autospec apparatus with NBA (3-nitrobencylic alcohol) as the matrix. Melting points were taken using a Gallenkamp apparatus and were left uncorrected. Gas chromatography–mass spectrometry (GC-MS) analysis was carried out on a chromatograph model Varian CP3800 with an ion-trap mass spectrometer model Saturn 2000 and under the following conditions: CP-Sil 8 low bleed/MS capillary column. The injector temperature was kept isothermal at 270°C; initial split conditions *on*; 0.01 min *off* and 5 min *on* with a split ratio 1:50; the oven was set at 50°C for 5 min, and then ramped at 15°C min^{-1} to 250°C and held for 10 min (total run time of 28.33 min for each sample); flux of 1 mL min^{-1}; mass detector in the EI mode (the m/*z* range was 20–400).

Results and discussion

Chemical analysis of the mycelium

Each box (2 L) of culture broth yielded 39.20–51.00 g fresh mycelium, corresponding to 3.99–5.56 g dry matter. The total wet mass of mycelium resulting from the sum of yields was 1,110.65 g, which produced, after IR desiccation, 118.00 g dry matter. The crude extract was obtained by maceration in CH_2Cl_2 (×3, 24 h) and CH_3OH (×3, 24 h) at room temperature. After filtration, evaporation and vacuum desiccation, 10.85 g brown oil was obtained. The whole extract was fractionated by polarity in a liquid-liquid extraction system, according to a modified version of the Kupchan method (Kupchan et al. 1973). Process flow diagrams (Fig. 1S) can be found in the supplementary electronic material, as shown in the Online Resource 1. Each one of the fractions was screened using chromatography (Column Chromatography, Size-

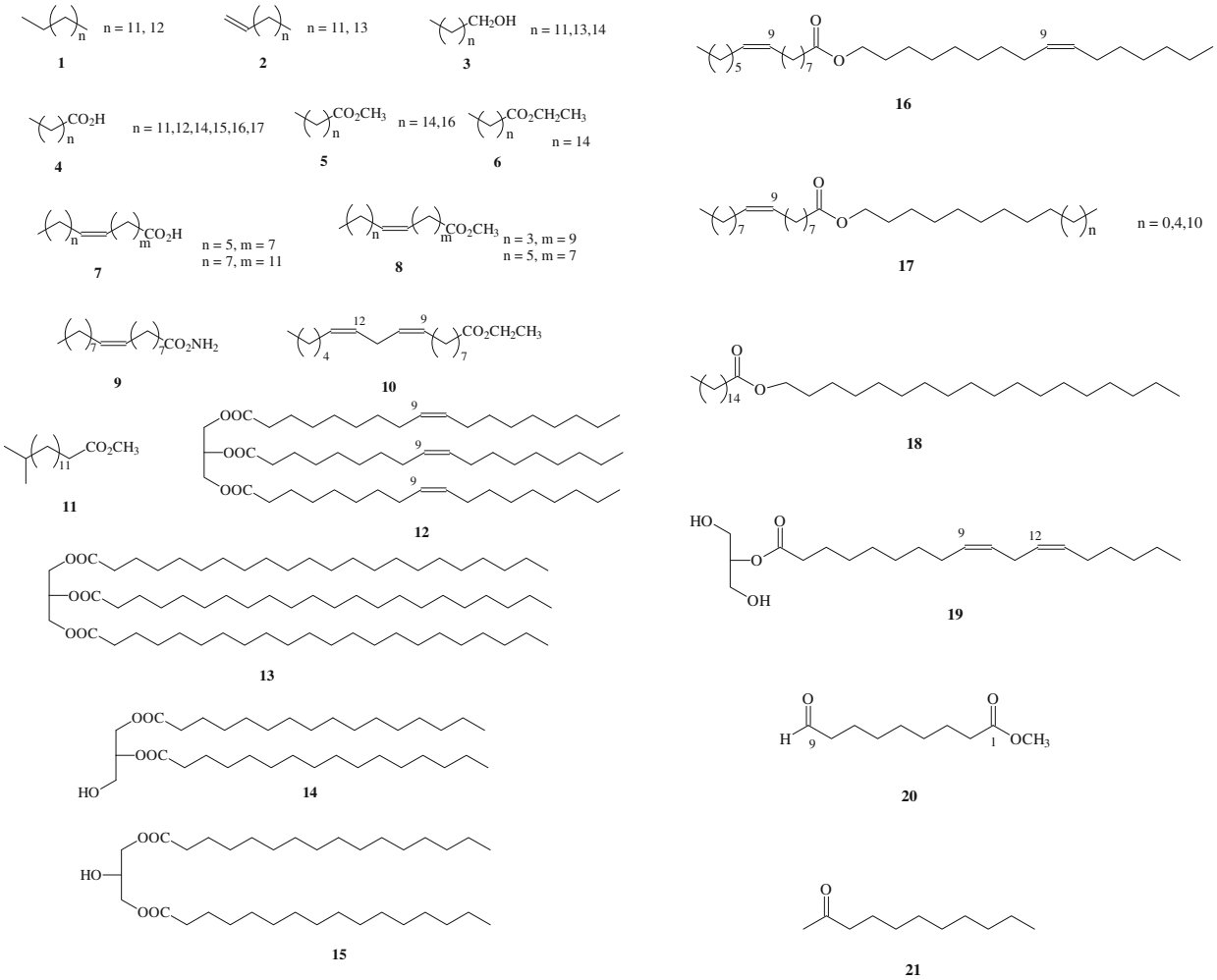

Fig. 1 Lipidic compounds identified in *Paecilomyces variotii*

exclusion Chromatography, Thin-layer Chromatography) and analysed using either GC-MS analysis for the volatile compounds, or by spectroscopy (NMR, MS, IR), identifying the following classes of compounds:

Volatile organic compounds

Volatile compounds were isolated by GC-MS (Table 1), and characterised and classified by structural criteria (Figs. 1, 2), as follows: *n*-alkanes (**1**), 1-alkenes (**2**), 1-alkanols (**3**), saturated (**4**) and unsaturated (**7**) free fatty acids, fatty acid amides (**9**), saturated (**5**, **6**) and unsaturated (**8**, **10**) fatty acid methyl and ethyl esters, saturated triglycerides (**13**) and diglycerides (**14**, **15**), unsaturated monoglycerides (**19**), wax esters (**16**, **17**, **18**), lipid catabolites (**20**), straight-chain terpenes (**22**, **23**) and a carbonilic compound (**21**). Additional experimental data are given in Online Resource 2.

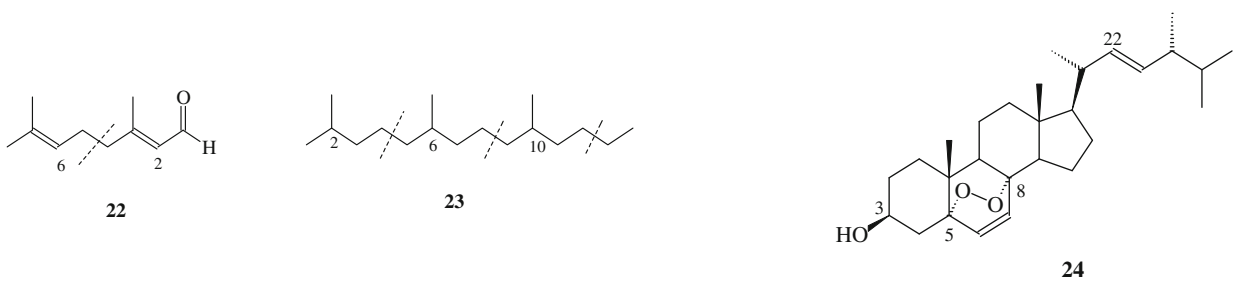

Fig. 2 Terpenoids identified in *P. variotii*

Table 2 ^{13}C-NMR and ^{1}H-NMR spectra ($CDCl_3$, 400 MHz) of ergosterol peroxide (**24**) isolated from *P. variotii*

C	δ (^{13}C)	δ (^{1}H)
18	12.890	0.771 (3H, s)
28	17.579	0.863 (3H, d, J=6.8 Hz)
19	18.185	0.839 (3H, s)
21	19.660	0.954 (3H, d, J=6.4 Hz)
26	19.970	0.772 (3H, d, J=6.8 Hz)
27	20.653	0.788 (3H, d, J=6.4 Hz)
11	20.902	*
15	23.417	*
4	28.665	*
16	29.721	*
2	30.140	*
25	33.075	*
1	34.705	*
10	36.972	*
12	39.363	*
20	39.767	*
24	42.794	*
13	44.580	–
9	51.101	*
14	51.707	*
17	56.209	*
3	66.504	3.94 (1H, m)
8	79.453	–
5	82.170	–
7	130.784	6.46 (1H, d, J=8.60 Hz), AB system
23	132.337	5.12 (1H, dd, J=7.5; 15.2)
22	135.240	5.08 (1H, dd, J=8.0; 15.2)
6	135.442	6.20 (1H, d, J=8.60 Hz), AB system

*3.45-1.15 (23 H, m)

Occurrence of branched chain fatty acid methyl esters

Methyl-branched fatty acids are distributed widely in nature (Nechev et al. 2002). It is now known that they are formed by the selective incorporation of methylmalonyl-CoA, catalysed by the fatty acid synthetase enzyme, and that this bioenergetic pathway is characteristic of bacteria that produce relatively high concentrations of these iso-methyl-branched fatty acids, which can therefore act as molecular markers for the organic matter produced by the bacteria (Kaneda 1991). Therefore, the identification of 14-methylpentadecanoic acid methyl ester (**11**) in *P. variotii* is indirect evidence of the presence of the *Mycobacterium* genus associated with this fungus (Chou et al. 1996). Apart from the GC-MS fingerprint, the iso-methyl-substitution proposed in **11** was also confirmed by the relatively intense fragment ion peak at M^+-43 (m/*z* 227) observed using GC-MS, together with the decrease in intensity of the M^+-29 (m/*z* 241) fragment (Andersson 1978).

Non-volatile organic compounds

The non-volatile organic extract obtained from the mycelium was fractionated and analysed by spectroscopy. The 5α, 8α-epidioxiergosta-6, 22-dien-3β-ol (**24**) compound, usually denominated ergosterol peroxide, was isolated in its purest form. Its structural elucidation was based on spectroscopic data (^{1}H-NMR, ^{13}C-NMR, DEPT_135, ^{1}H-^{1}H-COSY, HSQC and HMBC), and the complete assignation of the proton and carbon NMR spectra is reported in Table 2. Although this compound has been isolated previously from *Paecilomyces tenuipes* (Nam et al. 2001) and from a wide range of organisms (Krzyczkowski et al. 2009), this is the first report of its presence in the *P. variotii* species.

This steroidal derivative exhibits a significant number of biological activities, including immunosuppressive, anti-inflammatory, anti-viral, and anti-tumor properties (Dembitsky 2008). In addition, ergosterol peroxide proves to be a potent inhibitor of lipid peroxidation, and exhibits higher anti-oxidant activity than the well-known antioxidants α-tocopherol and thiourea (Kim et al. 1999). Consequently, this lipophilic metabolite may possess significant geno-protective properties, thereby providing evidence for the potential use of *P. variotii* as a high-value nutraceutical ingredient. Ergosterol peroxide can be obtained from ergosterol by photosensitised oxygenisation with eosin (González et al. 1983; Windaus and Brunken 1928), and at present is extracted from the fungus *Pleurotus eryngii* to manufacture skin-lightening products (Lee and Jeon 2010).

The chemical characterisation of the more polar fraction of the *P. variotii* extract produced a polyhydroxilic compound, the ^{1}H-NMR spectra of which suggests a monosaccharide structure. Through the HPLC analysis of this fraction using a Shodex OHpak SB806 HQ column, it was deduced that it was made up fundamentally of D-(+)-glucose. In fact, a number of hexoses and pentoses are commonly found as constituents of fungal cell wall-polysaccharides (Leal 1994), and glucose is generally the major component detected in the exopolysaccharides from some *Paecilomyces* species (Lillo et al. 2007). Therefore, it is probable that, in the present work, this monosaccharide has been extracted from the broth in which the mycelium had grown.

Another compound isolated was triolein (**12**), which was obtained from one of the less polar fractions of the crude extract in the form of a pale yellow oil. Its identification was based on IR, ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H-COSY, ^{1}H-^{1}H-TOCSY (Fig. 3), HSQC and HMBC spectroscopic data. Although no accurate estimation was made in the present work, triolein was isolated in relative amounts (0.4 % DW of mycelium). In agreement with this finding, lipid analysis

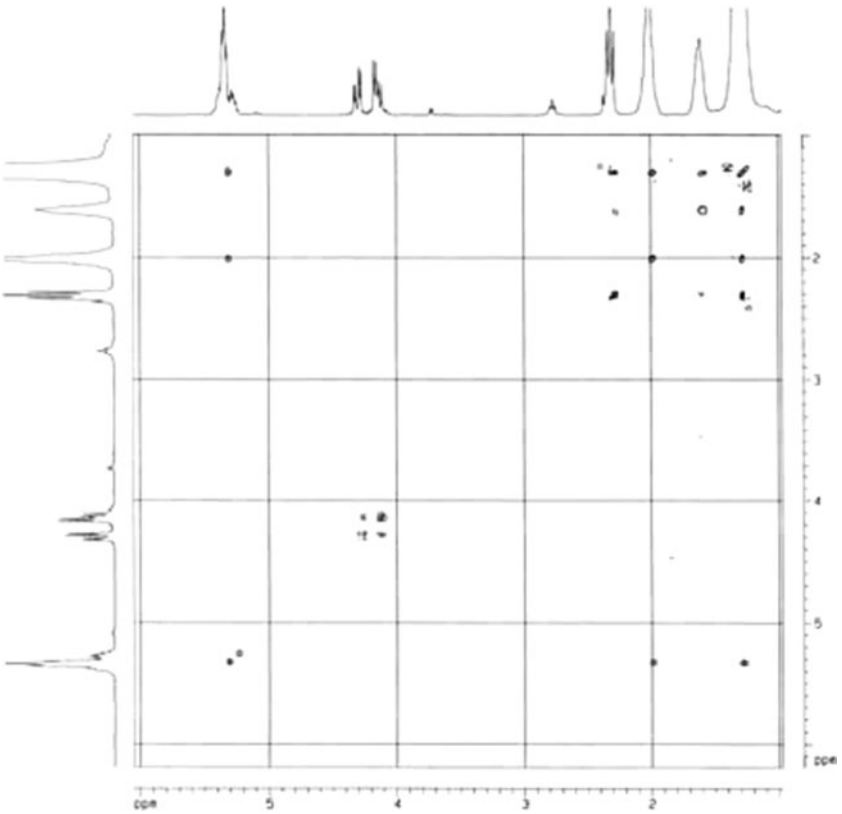

Fig. 3 ^{1}H-^{1}H-TOCSY spectrum of triolein (**12**) in *P. variotii*

carried out for another closely related fungus (*Paecilomyces tenuipes*) showed that the major components of the unsaturated esterified fraction were oleic (*cis* 18:1, *w*-9) and linoleic (18:2, *w*-6) acids, with the former predominant (Hong et al. 2007). In the same way, the total lipid crude extract 1.4% of the dry weight (DW) of mycelium obtained in the present work offered similar yields (1.3% DW of mycelium) to those previously reported by Lichtfield (1968) when the *P. variotii* was cultivated on sulphide waste liquid as a substrate.

Although a number of factors should affect either the fatty acid composition or the percentage of total lipids found in fungi (Dyal and Narine 2005), fatty acid analysis in the cultured *Paecilomyces variotii* indicates a limited capacity of lipid accumulation in this strain that may make it unsuitable for use as a source of bio-diesel. However, the characterisation of oleic acid as a major constituent of the unsaturated fatty acid fraction offers functional benefits of oxidative stability and nutritional attributes to this fungus. Triolein is known to be an important oil in the food industry, in dietetics and cosmetics, and can be extracted from various plant oils (Lisa and Holcapek 2008). The application of these filamentous fungi might play an important role, offering a new alternative source of high unsaturated fatty acids, such as triolein, for functional food.

Absence of mycotoxins

Although there are few studies on the chemistry of Ascomycota, it is widely accepted that different metabolite profiles can result from each lineage or fungal strain and, also as the result of different culture conditions. However, and using the present methodology of biomass production, we were unsuccessful in our search for possible toxic bioactive compounds in the mycelium, or even intermediary metabolites involved in a biogenetic route that could give rise to these substances.

Consistent with this observation, no single non-volatile organic compound was found that could bind biogenetically with the mycotoxins previously described in the literature for the *Paecilomyces* genus, e.g., Brefeldin A and Leucinostatins (Radics et al. 1987; Wang et al. 2002). This suggests the potential use of this strain for food purposes, both animal and human.

Conclusions

In summary, an unusual strain of *Paecilomyces variotii* was found where no toxin production has been detected. This study identified compounds that had already been reported in the literature but also detected new ones for this fungus. The results, although preliminary, support the idea that the metabolic profiles found in the mycelium suggest its potential use as therapeutic agent and natural source to be used in the production of nutraceuticals and functional foods. Without doubt, these filamentous fungi represent an area with enormous exploratory potential for new fermentation products.

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