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RESEARCH PAPER

Evaluation of antioxidant activity of the fermented product from the biotransformation of R-(+)-limonene in solid-state fermentation of orange waste by *Diaporthe* sp.

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Abstract Through solid-state fermentation, the endophytic fungus *Diaporthe* sp. biotransformed the compound R-(+)-limonene, a great quantity of which is present in orange waste. The fermented orange waste was evaluated to determine its antioxidant potential. Mass spectrometry identified several biotransformation products, which were quantified by gas chromatography. The fermentation process yielded compounds such as limonene-1,2-diol, α-terpineol, (−)-carvone, α-tocopherol, dihydrocarveol and valencene, most of which have already been associated with antioxidant activity. The highest concentration of limonene-1,2-diol produced was 3.02 g/kg of dry substrate and 0.72 g/kg of α-terpineol. The DPPH, ORAC and CUPRAC methods were employed to analyze the antioxidant activity comparing the orange waste and the fermented orange waste. According to the results obtained using the DPPH method, the fermented media extract represented 20.17% of antioxidant activity, compared to 12.1% of the orange waste extract, while from the ORAC method analysis the results were 24,011.39 μmolTE/g, obtained from the fermented extract in comparison to 5226.45 μmolTE/g from the orange waste. The results from the CUPRAC method analysis were 538.05 mg TE/g of dry extract, from the fermented extract in comparison to 168.27 TE/g of dry extract, from the orange waste. These results prove that the fermentation process increased the antioxidant potential of the orange waste.

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Introduction

The 2017/18 forecast for global orange production estimated a yield of 49.3 million tons, while the global forecast for orange juice production in the same year was approximately 1.7 million metric tons. In Brazil, the orange production is forecast to 17.3 million tons, while the orange juice production is estimated to 1.2 million metric tons. Brazil accounts for three-quarters of global orange juice exports (USDA, 2018). The orange residue in juice processing, composed of seed, flesh and skin, comprises approximately 50% of the fruit (Crizel, Jablonski, Rios, Rech, & Flôres, 2013).

Solid-state fermentation (SSF) has emerged as a potential technology for the manufacture of microbial products such as animal feed, fuel, food, industrial chemicals and pharmaceutical commodities. Utilizing agro-industrial residues as substrates in SSF processes lends unexpected value – a second life, as it were – to these conventionally under – or non-utilized residues (Pandey, 2003). There has been research testing microorganisms in submerged fermentation processes that use orange oil (Badee, Helmy, & Morsy, 2011; Maróstica Júnior and Pastore, 2007a). However, other authors have used an alternative approach to finding various microbial products that rely on the orange peel and bagasse (Mantzouridou, Paraskevopoulou, & Lalou, 2015; Pourbafrani, Forgács, Horváth, Niklasson, & Taherzadeh, 2010; Santi et al., 2010; Yang, Ma, & Lee, 2013).

Yet no documented research exists on the production of limonene derivatives through solid-state fermentation and research on the biotransformation of limonene via natural media is also scarce. Due to the substantial amount of orange waste available and the advantages of relying on biotechnological and biotransformation processes, the effective utilization of this residue reflects great potential. Citrus fruits have high concentrations of limonene (Arce, Pobudkowskal, Rodríguez, & Soto, 2007) which this makes them an ideal source of limonene for use in biotransformation processes.

Bacteria have been widely used in bioengineering, but the use of organisms like endophyte fungi has not been fully explored in the literature (Wang & Dai, 2011). Endophytic fungi are highly conducive to the efficient production of several groups of compounds with a variety of applications (Qadri et al., 2015). However, few articles have been published on rendering significant concentrations of aroma compounds and no articles have been published about generating significant concentrations of limonene derivatives via a biotransformation process. The biotransformation of terpenes is noteworthy because it facilitates the production of enantiomerically pure flavor compounds and fragrances, even under mild reaction conditions (Carvalho & Fonseca, 2006).

Beyond the attention paid to the sensorial properties, many studies identify such bioactive properties of orange peel and essential oil as mechanisms of antifungal activity (Velázquez-Nuñez, Avila-Sosa, Palou, & López-Malo, 2013) and anti-inflammatory effects (Gosslau, Chen, Ho, & Li, 2014), as well as antioxidant (Chen, Chu, Chyau, Chu, & Duh, 2012; Lu et al., 2012), antitumoral (Kaur & Kaur, 2015) and pesticide activity (El-Akhal, Lalami, & Guemmouh, 2015).

Table 1 Physicochemical composition of the orange waste.

Parameters	Content (%)
Moisture	14.2 ± 0.14
Reducing sugar	10.13 ± 0.99
Total sugar	21.13 ± 1.57
Protein	5.69 ± 0.21
Ashes	3.20 ± 0.12
Lipid	2.89 ± 0.15

Limonene and its derivatives have many additional bioactive properties related to antioxidant and antigenotoxic activities (Bacanli, Basaran, & Basaran, 2015), as well as the inhibition of angiogenesis, metastasis and cell death in human colon cancer cells (Murthy, Jayaprakasha, & Patil, 2012). Although there is clearly a wealth of compelling properties, this article focuses on the increased antioxidant capacity of the fermented product with respect to the orange residue.

This study aims to offer an account of the biotransformation of limonene through solid-state fermentation by the endophytic fungus *Diaporthe* sp. cultivated on a natural medium composed of orange waste. With regard to the aforementioned antioxidant activity, the following discussion furnishes a comparison between the orange extract and the fermented extract.

Material and methods

Microorganism

Diaporthe sp. was initially selected for its ability to resist and metabolize limonene (Bier, Poletto, Soccol, Soccol, & Medeiros, 2011). The strain is maintained in the Culture Collection of the Laboratory of Biotechnological Processes at the Federal University of Paraná (LPBI-UFPR) and registered at the World Data Center for Microorganisms (WDCM). The fungus *Diaporthe* sp. was originally isolated from the bark of *Pinus taeda*, after which its rDNA ITS sequence was deposited in GenBank under the accession number *Diaporthe* sp. KY113119.

Orange waste

The orange waste (peel and bagasse) used for the fermentation process was obtained in a canteen located at the Federal University of Paraná (Curitiba, PR, Brazil). The solid substrate was cut into pieces *in natura* and dried at a temperature of 60 °C, in an oven with air circulation, to prevent storage-related contamination and facilitate subsequent milling. The dried material was milled and classified granulometrically between 0.8 and 3 mm. The essential oil content in the orange residue was previously determined as 5.3% (Bier et al., 2016). Its main terpene composition consisted of 95.32% of R-(+)-limonene, 0.4% of β-pinene and 0.24% of α-pinene. The physicochemical composition (Table 1) of the orange waste (peel and bagasse) was determined according to the analytical standards of Instituto Adolfo Lutz (IAL, 2008).

Inoculum preparation

Diaporthe sp. was cultivated in 250 mL-capacity Erlenmeyer flasks containing 50 mL of potato dextrose agar (PDA) and incubated at 30 °C for 168 h. The mycelial suspension was prepared by adding 25 mL of sterile distilled water under magnetic stirring for 10 min. *Diaporthe* sp. showed no spores. The inoculum volume was fixed at 3 mL for 40 mL of medium.

The medium was prepared by adding 10 mL of water/g of dry orange waste to a boiling water bath for 20 min. The extract was filtered and separated into Erlenmeyer flasks of 125 mL containing 50 mL in each.

The inoculum was grown over 5 days at 30 °C, under agitation at 120 rpm, in a natural orange medium to which ammonium sulfate (5 g/L) was added. The medium was autoclaved for 15 min at 121 °C. Five mL of the fungi suspension was added to the solid state fermentation medium.

Solid-state fermentation

Experiments were carried out according to the process described by Soccol, Medeiros, and Bier (2014). The orange peel containing residue and orange bagasse was used as a substrate for solid-state fermentation, due to its high content of limonene (5.08%). Twenty g of dried orange residue was placed into Erlenmeyer flasks of 250 mL. The water content was adjusted to 80% moisture. The initial pH of the medium was adjusted to 6.0. The particle-size distribution of the medium was a mixture (1:1, w/w) of particles from 0.8 to 2 mm and 2 mm to 3 mm. The culture medium was sterilized by autoclaving at 121 °C for 15 min. The fermentation occurred over 7 days at 30 °C.

Optimization

Various experimental designs were used to study the main factors that influence the biotransformation of limonene by SSF. The experimental designs were developed using the software, Statistica® version 7.0. Tests were carried out to study the effects of pH, inoculum ratio and granulometry of the substrate.

An experimental design (2^3) with three factors and three replicates of the central point was applied. Due to its importance, as reported in the literature, the following independent variables were studied: pH, inoculum ratio and particle size. Humidity was maintained at 80%, due to the microorganism's inefficient growth capacity at moisture levels below 80%. The pH levels studied were: 4 (−1), 5 (0) and 6 (+1), a volume inoculum of 3 mL (−1), 5 mL (0) and 7 mL (+1) and a lower granulometry ranging from 0.8 to 2 mm (−1), a mixture of 0.8 to 2.0 and 2.0 to 3.0 mm (0) and from 2.0 to 3.0 mm (+1). A second fractional experimental design, with three factors and three levels (3^{3-1}) was implemented with the same variables, using the central-point values as levels.

Extraction procedure

The extraction of terpenoids from the orange residue was performed in portable extractor equipment (Bier et al., 2016). Liquefied petroleum gas (LPG), a mixture comprised of butane and propane, was used as the extractor solvent. The composition of the gas was 25% ± 5% propane and 75% ± 5% (w/w) isobutane + n-butane. The sample amount was 22 g. Each extraction cycle used 15 g of LPG (Volcano Isqueiros Ltda., SP, Brazil). The exposure time of the sample material was set to 20 min per cycle at 35 °C. All assays were performed in triplicate.

Volatile compounds analysis

The volatile compounds present in the extracts were analyzed by gas chromatography (GC), as described by Bier et al. (2016). The equipment used was a GC-17A gas chromatograph from Shimadzu, with a flame ionization detector, HP-5 column (30 m × 0.32 mm) and nitrogen as the carrier gas. The injector temperature was 250 °C, the detector temperature was 280 °C and, using a temperature program that set the initial oven temperature at 40 °C for 2 min, increasing 5 °C/min until it reached 150 °C. From 150 °C to 170 °C, there was an increase of 10 °C/min and an increase thereafter of 30 °C/min, until reaching the final temperature of 250 °C, which temperature was maintained for 2 min. A split ratio of 1:40 was used. The extracts were diluted in n-hexane (>95%). The results were expressed as percentage of peak area of product relative to the peak area of (R)-(+)-limonene (97% Sigma). The analysis of the extracts of solid-state fermentation was determined in g/kg substrate, based on a standard curve of (1S-2S-4R)-(+)-limonene-1,2-diol (≥97% Aldrich).

The identification of the volatile compounds was performed in a gas chromatograph, coupled with a mass detector (GC–MS) Shimadzu TQ 8040 equipped with a column DB-5 (30 m × 0.32 mm) and triple quadrupole detector. The auto-sampler was an AOC-5000 and the volume injected was 1 μL. The solvent cut was set at 3 min. The scan ranged from 30 to 400 *m*/*z*. The injector and column parameters were the same as those used for the gas chromatographic analysis. The identification of extract components was evaluated by a comparison with the MS standards of the National Institute of Standard and Technology (NIST, 2014). All analyses were performed for each of the triplicate assays. The results were also validated by verification using retention indices in gas chromatography of the standards of (R)-(+)-limonene (97% Sigma), D-carvone (Sigma-Aldrich, ≥96%), (1S-2S-4R)-(+)-limonene-1,2-diol (Aldrich, ≥97%), terpineol (Aldrich, 99.5%) and (−)-carveol (Sigma-Aldrich, 97%).

Determination of antioxidant activity using the DPPH method

The antioxidant activity of the fermented samples (100 μL), orange waste extract, R-(+)-limonene and (1S-2S-4R)-(+)-limonene-1,2-diol were determined using 1.4 mL of 0.1 mM of DPPH (2,2-diphenyl-1-picrylhydrazyl, Sigma-Aldrich) solution. The DPPH solution was prepared in methanol and used as the reagent to establish the BHA (butylated hydroxyanisole) and ascorbic acid standard curves. The IC50 of the extract obtained from the fermented orange residue was determined. The other samples were compared at the concentration of 4%. The analyses were performed

in triplicate in a spectrophotometer at 517 nm. A sample containing only methanol was used as the blank.

Determination of the antioxidant activity using the CUPRAC method

The CUPRAC (cupric ion reducing antioxidant capacity) method was adapted from the test described by Apak et al. (2007). The absorbance was measured at 450 nm using the spectrophotometer. The antioxidant potential of R-(+)-limonene, (1S-2S-4R)-(+) limonene-1,2-diol, orange waste extract and the fermented extracts was measured with Trolox equivalents (TEAC values). The statistical significance was verified by the Tukey's method for multiple comparison.

Determination of the antioxidant activity using the ORAC method

The Oxygen Radical Absorbance Capacity Assay (ORAC) was performed according to Zulueta, Esteve, and Frígola (2007). The analysis was performed in a 96-well microplate with the addition of 50 μL of the samples (R-(+)-limonene, limonene-1,2-diol, orange waste extract and fermented extract), 50 μL of fluorescein sodium salt (Sigma) at 78 nM in phosphate buffer 75 mM, pH 7.0 and 25 μL of AAPH (2,2′-Azobis(2-amidinopropane) dihydrochloride, 97%, Aldrich) – 221 mM in phosphate buffer, daily prepared. The microplate reader used was a TECAN *infinete*® 200MPRO. The sample was exposed to an excitation wavelength of 485 nm and the emission wavelength was 535 nm for 30 min (at 60-second intervals). The reaction occurred at 37 °C ± 0.5. The Trolox standard was prepared at concentrations ranging from 3.25 μmol/L to 100 μmol/L. The data were analyzed using the Microsoft Excel application. The area under the curve (AUC) was calculated as:

$$\text{AUC} = 0.5 + \frac{f1}{f0} + \ldots \frac{fi}{f0} + \ldots + \frac{f29}{f0} + 0.5\left(\frac{f30}{f0}\right)$$

where *f*0 is the initial fluorescence reading at 0 min and *fi* is the fluorescence reading at time *i*. The net AUC is obtained by subtraction of the AUC of the blank from that of the sample. The relative Trolox equivalent ORAC value is calculated as:

$$\text{Relative ORAC value} = \text{CTrolox}\left[\frac{(\text{AUCsample} - \text{AUCblank}) \cdot k}{(\text{AUCTrolox} - \text{AUCblank})}\right]$$

where CTrolox is the concentration of Trolox and *k* is the sample dilution factor.

Determination of total phenolics

Total phenolic content was measured using Folin–Ciocalteu (Sigma-Aldrich, 2 mol/L) spectrophotometric method (Song et al., 2010) using gallic acid (Vetec 98%) for the calibration curve. All tests were performed in triplicate, and the results were presented as gallic acid equivalents (mg/g extract). The sample was diluted to 1:250, and 0.5 mL was reacted with 0.2 mol/L of the Folin–Ciocalteu reagent for 5 min. Afterwards, 2 mL of sodium carbonate (7.5%, w/v) was added to the reaction mixture. The absorbance readings were performed at 760 nm after incubation at 37 °C for 60 min.

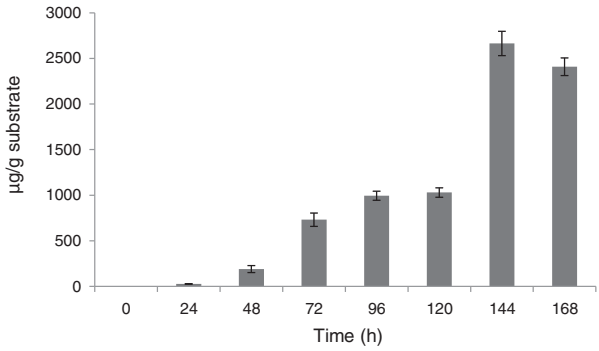

Figure 1 Limonene-1,2-diol concentration during 168 h of solid state fermentation of orange waste by *Phomopsis* sp.

Results and discussion

Solid-state fermentation of orange waste

The limonene biotransformation by *Diaporthe* sp. was performed over seven days in order to determine the best time for production of limonene derivatives (Fig. 1). The highest concentration of limonene-1,2-diol was achieved after 144 h of fermentation (2.66 g/kg substrate). Limonene-1,2-diol or limonene-glycol is a colorless to very-slightly-yellow oil, with a cool minty aroma during consumption, with the odor and/or flavor used in mint (Burdock, 2010). Other compounds produced in significant amounts were α-terpineol (0.44 g/kg of substrate), trans-carveol (0.13 g/kg of substrate) and cis-carveol (0.21 g/kg of substrate). It is important to note that 144 h may be regarded as a lengthy fermentation period regarding the limonene biotransformation process. *Penicillium digitatum* was able to convert limonene into (R)-(+)-α-terpineol after only 8 h (Adams, Demyttenaere, & De Kimpe, 2003). However, Demyttenaere, Belleghem, and De Kimpe (2001) described the bioconversion to limonene-1,2-diol as a slow process, occurring at the end of a five-day experiment. Both studies were performed, however, using submerged fermentation at low concentrations of limonene, while the concentration of the limonene in the orange waste media with 80% of humidity is higher than 1%. In our study, significant concentrations of limonene-1,2-diol were initially achieved after 48 h (191.60 μg/g substrate) and 72 h (732.96 μg/g substrate) of fermentation. Few studies concerning biotransformation experiments using endophytes have been reported, rendering some of its fermentation features still unknown. The behavior cannot be fully compared once the above mentioned biotransformation processes occur using submerged fermentation.

After factorial-design studies, including pH, particle size and inoculum volume, the concentration of α-terpineol increased from 0.44 g/kg to 0.72 g/kg when the granulometry was at 2–3 mm, pH 6.0 and inoculum size of 3 mL. The highest production of limonene-1,2-diol also increased and

Table 2 Major volatile compounds identified by gas chromatography–mass spectrometry (GC–MS) in the orange waste extract and fermented orange waste by *Phomopsis* sp.

Compound	CAS	OWE[1]	FE[2]	MS fragments
n-Butyl acetate	123-86-4	97%	–[3]	43, 56, 87, 115
α-Pinene	80-56-8	95%	88%	39, 53, 93, 107, 136, 154
β-Phellandrene	555-10-2	90%	–	41, 65, 93, 121, 136
Sabinene	3387-41-5	–	94%	41, 77, 93, 121, 136
β-Myrcene	123-35-3	95%	95%	41, 69, 93, 121, 136
β-Pinene	127-91-3	96%	90%	41, 69, 93, 121, 136
3-Carene	13466-78-9	96%	–	41, 67, 93, 121, 136
Cis-β-ocimene	3338-55-4	–	92%	41, 77, 93, 105, 136
Limonene	5989-27-5	95%	95%	41, 68, 93, 121, 136
n-Octanol	111-87-5	–	95%	41, 56, 84, 112, 129
2-Phenylethanol	60-12-8	–	97%	39, 65, 91, 122
Linalool	78-70-6	94%	92%	41, 71, 93, 121, 136, 154
Trans-p-mentha-2,8-dien-1-ol	7212-40-0	92%	–	43, 67, 79, 109, 134, 152
Cis-p-mentha-2,8-dien-1-ol	22771-44-4	92%	91%	43, 67, 91, 109, 137, 152
trans-Isopulegone	29606-79-9	–	91%	41, 67, 93, 109, 134, 152
(−)-Trans-isopiperitenol	74410-00-7	–	94%	41, 69, 84, 108, 134, 152
α-Terpineol	98-55-5	94%	91%	43, 59, 93, 95, 121, 139
(R)-(+)-Verbenone	18309-32-5	83%	–	39, 55, 91, 107, 135, 150
Cyclohexyl isothiocyanate	1122-82-3	86%	–	41, 55, 83, 109, 141
Neo-dihydrocarveol	18675-34-8	–	88%	41, 55, 93, 107, 136, 154
Dihydrocarveol	619-01-2	–	87%	41, 68, 93, 107, 136, 154
Limonene dioxide	96-08-2	–	88%	43, 67, 79, 107, 123. 153, 168
D-(+)-Carvone	2244-16-8	92%	–	39, 54, 82, 108, 135, 150
L-(−)-Carvone	6485-40-1	–	89%	39, 54, 82, 109, 135, 150
Cis-linalool oxide	5989-33-3	–	86%	43, 59, 94, 111, 137, 155
Trans-ascaridol glycol	21473-37-0	–	86%	43, 55, 81, 109, 127, 152
Limonene-1,2-diol	1946-00-5	–	96%	43, 71, 82, 108, 137, 152
γ-Muurolene	30021-74-0	88%	–	41, 67, 91, 119, 133, 161, 189, 204
(−)-α-Copaene	3856-25-5	97%	–	41, 55, 91, 119, 133, 161, 189, 204
β-Copaene	18252-44-3	91%	–	41, 55, 91, 105, 133, 161, 189, 204
β-Caryophyllene	87-44-5	96%	–	41, 69, 93, 105, 133, 161, 189, 204
Alpha-Caryophyllene Humulene	6753-98-6	92%	–	41, 55, 93, 121, 147, 161, 189, 204
Germacrene D	23986-74-5	94%	–	41, 67, 91, 105, 133, 161, 189, 204
Valencene	4630-07-3	–	92%	41, 67, 91, 105, 133, 161, 189, 204
Naphthalene	91-20-3	94%	–	41, 67, 91, 105, 133, 161, 204
α-Muurolene	31983-22-9	94%	–	41, 77, 93, 105, 133, 161, 189, 204
Azulene	275-51-4	91%	–	41, 55, 93, 107, 135, 161, 189, 204
Butylated hydroxytoluene	128-37-0	86%	–	41, 57, 91, 105, 145, 161, 177, 205
β-Cadinene	523-47-7	90%	–	41, 55, 81, 119, 134, 161, 189, 204
Caryophyllene oxide	1139-30-6	88%	–	43, 69, 79, 109, 135, 149, 177, 220
Trimethylsilyl ester of tetradecanoic acid	18603-17-3	92%		43, 73, 83, 117, 129, 159, 185, 201, 241, 257, 285, 300
Methyl palmitate	112-39-0	95%	–	43, 74, 87, 115, 143, 171, 185, 199, 227, 256, 270
Dioctyl isophthalate	117-84-0	–	92%	41, 70, 83, 112, 149, 167, 261, 279
L(+)-Ascorbic acid	50-81-7	89%	89%	43, 57, 85, 115, 129, 157, 185, 213, 239, 256
Ethyl palmitate	628-97-7	92%	91%	43, 55, 88, 115, 143, 157, 199, 239, 284
Linoleic acid	60-33-3	94%	–	43, 61, 81, 109, 136, 150, 178, 220, 263, 279, 294
Methyl octodeca-9,12-dienoate	2462-85-3	–	90%	41, 67, 81, 109, 136, 150, 178, 263, 294
Citric acid	77-92-9	93%	–	43, 57, 87, 112, 129, 157, 185, 213, 231, 259, 273, 305, 329, 343
α-Tocopherol	10191-41-0	–	88%	43, 57, 91, 121, 136, 165, 205, 430
Eicosane	112-95-8	94%	–	43, 50, 85, 113, 127, 155, 183, 211, 225, 253, 282

[1] Orange waste extract
[2] ermented media extract
[3] Compound not detected

occurred at pH 6.0, with a particle size of 0.8–2.0, and reached 3.02 g/kg of substrate after 144 h of fermentation.

This achievement of limonene-1,2-diol (3.02 g/kg) is superior to most reports of limonene-1,2-diol, which is typically the minor fermentation product (Demyttenaere & De Kimpe, 2001; Molina, Pinheiro, Pimentel, Dos Sanros, & Pastore, 2013), but also superior to reports of it, even as a major fermentation product. However, no production of α-terpineol or limonene-1-2-diol had previously been reported on solid-state fermentation.

Production of volatile compounds

Several volatile compounds were detected in the extract of the orange waste fermented by *Diaporthe* sp. Among them, (R)-(−)-carvone, α-terpineol, 1,6-dihydrocarveol, (−)-trans-isopiperitenol, limonene-1,2-diol and α-limonene diepoxide are particularly noticeable among the compounds identified via GC–MS analysis (Table 2). These compounds are the most important because they are limonene derivatives (Maróstica & Pastore, 2007b). There are major differences between the extracts of orange waste and fermented orange waste. For example, the high numbers of mono-, di- and triterpenoids, such as D-verbenone, 3-carene, β-cadinene, azulene, germacrene and naphthalene, present in the precursor waste reflects a stark contrast with the more complex derivatives, such as limonene-1,2-diol, cis-linalool oxide, limonene-diepoxide, trans-isopulegone and the main fermentation products cited above, observed in the fermented orange waste. Also, a substantial amount of esters and carboxylic acids (n-butyl acetate, propanoic acid, mirystic acid) can be observed in the waste extract precursor, while the fermented waste extract contains more alcohols (caprilic alcohol, dihydrocarveol and the ones already cited), as well as some interesting compounds with significant bioactive properties, like valencene, sabinene and α-tocopherol (Liu, Chen, Liu, Zhou, & Wang, 2012).

Antioxidant activity per DPPH

The antioxidant capacity of the fermented extract was established by comparing its activity to butylated hydroxyanisole (BHA) and ascorbic acid. Fig. 2 shows the antioxidant activity of the fermented extract relative to the blank.

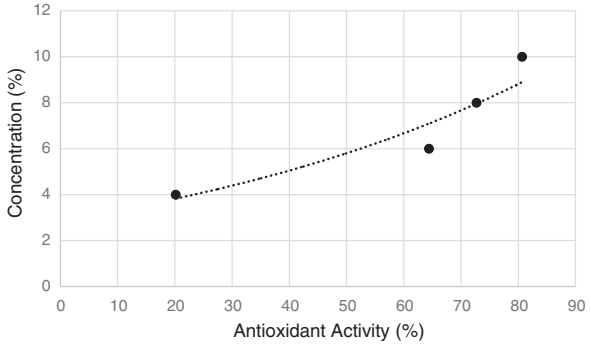

Figure 2 Antioxidant activity of the fermented orange waste extract in comparison to the blank.

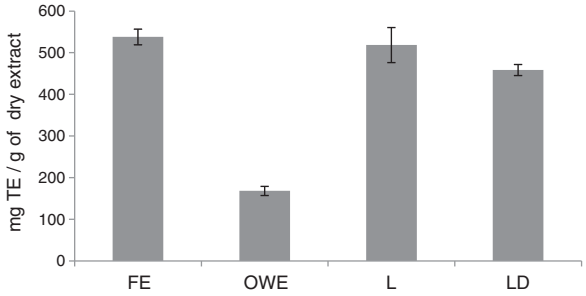

Figure 3 Antioxidant activity of the standards and the extracts by CUPRAC (mg TE/g dry extract). *Abbreviations*: FE, fermented orange waste extract; OWE, orange waste extract; L, limonene; LD, limonene-1,2-diol.

At a concentration of 5.79%, the orange waste extract (diluted at a ratio of 1:10) has the antioxidant capacity equivalent to 85.938 mmol of BHA or 107.277 mmol of ascorbic acid. Furthermore, the IC50 is 5.79% of the original extract.

The fermented orange waste extract was capable of reducing the stable, purple-colored radical DPPH, given the 80.68% inhibition, with an initial concentration of 10% (1:10). At 4%, the fermented orange waste extract presented 20.2% inhibition, while the (unfermented) orange waste extract (peel and bagasse) showed 12.1% inhibition at the same concentration. Sarrou, Chatzopoulou, Dimassi-Theriou, and Therios (2013) determined the scavenging activity of flowers, young leaves and peel oil of citrus as 53.98%, 22.79% and 19.29%, respectively, while the antioxidant potential of the essential oil of two *Citrus sinensis* specimens was 10.5% and 30%, respectively (Malhotra, Suri, & Tuli, 2016). At last, the antioxidant potential of the orange waste extracts fermented by *Diaporthe* sp. showed significant results, especially in comparison to the results observed from the orange oil. This proves that the compounds produced by the fungus (via solid-state fermentation) presented a higher antioxidant activity than their precursors.

The DPPH results for limonene and limonene-1,2-diol showed the latter compound to have a higher antioxidant capacity (5.67% ± 0.89) than limonene (3.65% ± 0.72), at a concentration of 4% (v/v). This result demonstrates that producing imonene-1,2-diol increases the antioxidant capacity of the extract because this compound has a higher potential than its more basic limonene precursor. However, it was not only the fermentation extract, but also the orange waste, that showed higher antioxidant capacity than the standards. Therefore, the antioxidant activity of the extracts may be attributed to other mono-, di- and sesquiterpenes present in the orange waste extract and the related derivatives present on the fermented extract.

Antioxidant capacity per CUPRAC

The antioxidant activity rendered by the CUPRAC method determined the fermented orange waste extract to have the highest antioxidant activity (538.05 mg TE/g of dry extract) (Fig. 3). Compared to the orange residue extract (168.27 mg TE/g of dry extract), the fermented orange waste extract shows more antioxidant activity by a factor

Table 3 Antioxidant capacity of the extracts and standards by the ORAC method.

Compound/material	Antioxidant capacity (μmolTE/g)
Orange waste extract	5226.45 ± 23.04
Fermented orange waste extract	24,011.39 ± 640.16
Limonene	5262.87 ± 33.0
Limonene-1,2-diol	18,220.89 ± 1080.88

of 3.2. The orange residue extract results square with the results obtained by Assefa, Ko, Moon, and Keum (2016), verifying that the activity of different citrus fruit extracts ranges from 16.8 to 208.7 mg TE/g of dry extract. The antioxidant potential shown by the fermented orange extract is also highly significant.

The results obtained from testing with limonene (519 mg TE/g) are slightly higher than those obtained with limonene-1,2-diol (459 mg TE/g), but this difference is not statistically significant ($p > 0.5$). The results obtained with the standards are similar to those obtained with the fermentation extract (FE) ($p > 0.1$). The similarity may indicate that the increased antioxidant potential of the fermented orange waste is not only due to the bioconversion of limonene to limonene-1,2-diol, but also to the variety of compounds in the fermented orange waste extract that show analogous antioxidant activity. The antioxidant activity of the orange waste extract is unambiguously low, despite its high limonene content of this extract. As such, it is reasonable to conclude that its other component compounds are likely to have contributed minimally to the antioxidant capacity.

Antioxidant capacity per ORAC

The antioxidant activity revealed through testing by the ORAC method (Table 3) indicates an increase in the antioxidant activity of the fermented orange waste extract (24,011.39 ± 640.16 μmolTE/g) relative to the natural potential of the orange waste extract (5226.45 ± 23.04 μmolTE/g). The values reflecting the orange waste extract activity, per the ORAC method, were higher than the values described by Jayaprakasha, Girennavar, and Patil (2008) with citrus fruits (2220.72 ± 22 μmolTE/g). This superior result is attributable to the efficiency of the extraction method employed.

According to this method, strong correlations exist between the antioxidant capacity of limonene and the orange waste extract, the fermented orange waste extract and limonene-1,2-diol and the production of limonene-1,2-diol from limonene. Limonene showed a potential of 5262.87 μmolTE/g, while limonene-1,2-diol had an ORAC value of 18,220.89 μmolTE/g.

The results obtained using the ORAC method square with the results obtained using the CUPRAC method. The increased antioxidant activity of the fermented orange waste relative to the orange waste extract is similar according to both testing methods. However, the CUPRAC method did not indicate the existence of a direct relationship between the production of limonene-1,2-diol and the antioxidant capacity of the fermented orange waste extract, because that method found antioxidant capacities for limonene and limonene-1,2-diol that were meaningfully similar.

Total polyphenol content

The results obtained using the Folin–Ciocalteu reagent show an increase of polyphenol content that reflects levels more than 8 times greater than the levels identified in the original orange extract (36.39 ± 1.97 mg gallic acid/g extract) relative to the fermented orange waste extract (271.33 ± 3.73 mg/g). Furthermore, the polyphenol content shows a direct correlation with the antioxidant activity obtained using the CUPRAC, ORAC and DPPH methods, all of which showed improved antioxidant capacity of the fermented orange waste extract.

Conclusion

This study presents a new perspective on the solid-state fermentation process as it bears on the use of endophytic fungus for a biotechnological process that relies on a natural waste medium. The transformation of limonene orange waste yielded limonene-1-2-diol and α-terpineol, indicating that using solid-state fermentation as process for the biotransformation of limonene and, possibly, other terpenoids is a subject particularly ripe for further study. It also confirms the increasing importance of endophyte fungi in various areas of the field of biotechnology.

All of the antioxidant tests conducted on the fermented orange waste extract yielded very positive results; all four methods showed a substantial increase in the antioxidant activity of the orange oil after the fermentation process. The scarcity of available information about the properties of limonene-1,2-diol remains problematic for the production and use of this limonene derivative in the flavor industry (on a broader scale). This paper thereby validates a series of new claims regarding the biotechnological biotransformation of limonene and offers a novel perspective regarding the prospect inherent in the demonstrated increase of antioxidant activity produced by using the fermented extract of *Diaporthe* sp.

Conflicts of interest

The authors declare no conflicts of interest.

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References

Adams, A., Demyttenaere, J. C. R., & De Kimpe, N. (2003). Biotransformation of (R)-(+)- and (S)-(−)-limonene to α-terpineol

by *Penicillium digitatum* – Investigation of the culture conditions. *Food Chemistry*, *80*, 525–534.

Apak, R., Güçlü, K., Demirata, B., Özyürek, M., Çelik, S. E., Bektaşoğlu, B., et al. (2007). Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay. *Molecules*, *12*, 1496–1547.

Arce, A., Pobudkowskal, A., Rodríguez, O., & Soto, O. (2007). Citrus essential oil terpenless by extraction using 1-ethyl-3-methylimidazolium ethylsulfate ionic liquid: Effect of the temperature. *Chemical Engineering Journal*, *133*, 213–218. http://dx.doi.org/10.1016/j.cej.2007.01.035

Assefa, A. D., Ko, E. Y., Moon, S. H., & Keum, Y.-S. (2016). Antioxidant and antiplatelet activities of flavonoid-rich fractions of three citrus fruits from Korea. *3 Biotech*, *6*, 1–10.

Bacanli, M., Basaran, A. A., & Basaran, N. (2015). The antioxidant and antigenotoxic properties of citrus phenolics limonene and naringin. *Food and Chemical Toxicology*, *81*, 160–170.

Badee, A. Z. M., Helmy, S. A., & Morsy, N. F. S. (2011). Utilisation of orange peel in the production of α-terpineol by *Penicillium digitatum* (NRRL 1202). *Food Chemistry*, *126*, 849–854.

Bier, M. C. J., Poletto, S., Soccol, V. T., Soccol, C. R., & Medeiros, A. B. P. (2011). Isolation and screening of microorganisms with potential for biotransformation of terpenic substrates. *Brazilian Archives of Biology and Technology*, *54*, 1019–1026.

Bier, M. C. J., Medeiros, A. B. P., De Oliveira, J. S., Côcco, L. C., Costa, J. L., & Soccol, C. R. (2016). Liquefied gas extraction: A new method for the recovery of terpenoids from agroindustrial and forest wastes. *The Journal of Supercritical Fluids*, *110*, 97–102.

Burdock, G. A. (2010). *Fenaroli's handbook of flavor ingredients*. pp. 2159. Boca Raton: CRC Press.

Carvalho, C. C. R., & Fonseca, M. M. R. (2006). Biotransformation of terpenes. *Biotechnology Advances*, *24*, 134–142.

Chen, Z. T., Chu, H. L., Chyau, C. C., Chu, C. C., & Duh, P. D. (2012). Protective effects of sweet orange (*Citrus sinensis*) peel and their bioactive compounds on oxidative stress. *Food Chemistry*, *135*, 2119–2127.

Crizel, T. M., Jablonski, A., Rios, A. O., Rech, R., & Flôres, S. H. (2013). Dietary fiber from orange byproducts as a potential fat replacer. *LWT – Food Science Technology*, *53*, 9–14. http://dx.doi.org/10.1016/j.lwt.2013.02.002

Demyttenaere, J. C. R., Belleghem, K. V., & De Kimpe, N. (2001). Biotransformation of (R)-(+)- and (S)-(−)-limonene by fungi and the use of solid phase microextraction for screening. *Phytochemistry*, *57*, 199–208.

Demyttenaere, J. C. R., & De Kimpe, N. (2001). Biotransformation of terpenes by fungi: Study of the pathways involved. *Journal of Molecular Catalysis B-Enzymatic*, *11*, 265–270.

El-Akhal, F., Lalami, A. E. O., & Guemmouh, R. (2015). Larvicidal activity of essential oils of *Citrus sinensis* and *Citrus aurantium* (Rutaceae) cultivated in Morocco against the malaria vector *Anopheles labranchiae* (Diptera: Culicidae). *Asian Pacific Journal of Tropical Disease*, *5*, 458–462.

Gosslau, A., Chen, K. Y., Ho, C., & Li, S. (2014). Anti-inflammatory effects of characterized orange peel extracts enriched with bioactive polymethoxyflavones. *Food Science and Human Wellness*, *3*, 26–35.

IAL. (2008). *Normas Analíticas do Instituto Adolfo Lutz*. pp. 1020. São Paulo: Instituto Adolfo Lutz.

Jayaprakasha, G. K., Girennavar, B., & Patil, B. S. (2008). Antioxidant capacity of pummelo and navel oranges: Extraction efficiency of solvents in sequence. *LWT – Food Science and Technology*, *41*, 376–384.

Kaur, J., & Kaur, G. (2015). An insight into the role of citrus bioactives in modulation of colon cancer. *Journal of Functional Foods*, *13*, 239–261.

Liu, K., Chen, Q., Liu, Y., Zhou, X., & Wang, X. (2012). Isolation and biological activities of decanal, linalool, valencene, and octanal from sweet orange oil. *Journal of Food Science*, *77*, 1156–1161.

Lu, Y., Zhao, S., Zhang, J., Zhang, H., Xie, Z., Cai, G., et al. (2012). Effect of orange peel essential oil on oxidative stress in AOM animals. *International Journal of Biological Macromolecules*, *50*, 1144–1150.

Malhotra, S., Suri, S., & Tuli, R. (2009). Antioxidant activity of citrus cultivars and chemical composition of *Citrus karna* essential oil. *Planta Medica*, *75*, 62–64.

Mantzouridou, F. T., Paraskevopoulou, A., & Lalou, S. (2015). Yeast flavour production by solid state fermentation of orange peel waste. *Biochemical Engineering Journal*, *101*, 1–8.

Maróstica Júnior, M. R., & Pastore, G. M. (2007a). Production of R-(+)-α-terpineol by the biotransformation of limonene from orange essential oil, using cassava waste water as medium. *Food Chemistry*, *101*, 345–350.

Maróstica Júnior, M. R., & Pastore, G. M. (2007b). Biotransformation of limonene: A review of the main metabolic pathways. *Química Nova*, *30*, 382–387.

Molina, G., Pinheiro, D. M., Pimentel, M. R., Dos Sanros, R., & Pastore, G. M. (2013). Monoterpene bioconversion for the production of aroma compounds by fungi isolated from Brazilian fruits. *Food Science and Biotechnology*, *22*, 999–1006.

Murthy, K. N. C., Jayaprakasha, G. K., & Patil, B. S. (2012). D-limonene rich volatile oil from blood oranges inhibits angiogenesis, metastasis and cell death in human colon cancer cell. *Life Science*, *91*, 429–439.

NIST, National Institute of Standards and Technology. (2014). *Mass Spectral Library and Search Software v.2.2 (NIST 2014/EPA/NIH)*. pp. 72. Gaithersburg, MD.

Pandey, A. (2003). Solid state fermentation. *Biochemical Engineering Journal*, *13*, 81–88.

Pourbafrani, M., Forgács, G., Horváth, I. S., Niklasson, C., & Taherzadeh, M. J. (2010). Production of biofuels, limonene and pectin from citrus wastes. *Bioresource Technology*, *101*, 4246–4250.

Qadri, M., Deshidi, R., Shah, B. A., Bindu, K., Vishwakarma, R. A., & Riyaz-Ul-Hassan, S. (2015). An endophyte of *Picrorhiza kurroa* Royle ex. Benth, producing menthol, phenylethyl alcohol and 3-hydroxypropionic acid, and other volatile organic compounds. *World Journal of Microbiology and Biotechnology*, *31*, 1647–1654.

Santi, G., Crognale, S., D'Annibale, A., Petruccioli, M., Ruzzi, M., Valentini, R., et al. (2010). Orange peel pretreatment in a novel lab-scale direct steam-injection apparatus for ethanol production. *Biomass and Bioenergy*, *61*, 146–156.

Sarrou, E., Chatzopoulou, P., Dimassi-Theriou, K., & Therios, L. (2013). Volatile constituents and antioxidant activity of peel, flowers and leaf oils of *Citrus aurantium* L. growing in Greece. *Molecules*, *18*, 10639–10647.

Soccol, C. R., Medeiros, A. B. P., & Bier, M. C. J. (2014). Bioprocesso para a produção de derivados de terpenos por *Diaporthe* sp. e *Rhodotorula mucilaginosa*. *Brazilian Patent*,. INPI BR 102014029813.

Song, F. L., Gan, R. Y., Zhang, Y., Xiao, Q., Kuang, L., & Li, H. B. (2010). Total phenolic contents and antioxidant capacities of selected Chinese medicinal plants. *International Journal of Molecular Sciences*, *11*, 2362–2372.

USDA, United States Department of Agriculture. (2018). *Citrus: World markets and trade*. pp. 13. Washington: Foreign Agricultural Service.

Velázquez-Nuñez, M. J., Avila-Sosa, R., Palou, E., & López-Malo, A. (2013). Antifungal activity of orange (*Citrus sinensis* var. Valencia) peel essential oil applied by direct addition or vapor contact. *Food Control*, *31*, 1–4.

Wang, Y., & Dai, C. C. (2011). Endophytes: A potential resource for biosynthesis, biotransformation, and biodegradation. *Annals of Microbiology*, *61*, 207–215.

Yang, F. C., Ma, T. W., & Lee, Y. H. (2013). Reuse of citrus peel to enhance the formation of bioactive metabolite-triterpenoid in solid-state fermentation of *A. cinnamomea*. *Biochemical Engineering Journal*, *78*, 59–66.

Zulueta, A., Esteve, M. J., & Frígola, A. (2007). ORAC and TEAC assays comparison to measure the antioxidant capacity of food products. *Food Chemistry*, *114*, 310–316.