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Sequential fungal fermentation-biotransformation process to produce a red pigment from sclerotiorin

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ABSTRACT

The fungus *Penicillium sclerotiorum* produces sclerotiorin, an orange compound closely related to the useful food coloring pigments produced by *Monascus* species. The high productivity, together with several biological activities reported for sclerotiorin highlights its potential application in food industry. In this work, sclerotiorin was obtained as the major metabolite produced in liquid fermentation by *P. sclerotiorum* standing for 30% of the fungal dry extract. Modulation of sclerotiorin color was accomplished by biotransformation using *Beauveria bassiana* generating a red derivative with 13.8% yield. Color modification was caused by fungal-mediated substitution of oxygen by nitrogen in the pyrone ring changing the molecule's chromophore. A derivative, 1-methyl sclerotiorin was synthesized from sclerotiorin using diazomethane and fed to *B. bassiana*. In this case, substituent at C-1 avoided heteroatom substitution. Sclerotiorin derivatives obtained in the present show the great potential of sclerotiorin derivatives as food colorants.

1. Introduction

The increased use of filamentous fungi and their metabolites in food industry nowadays occurred due to the prompt adaptation of fungal metabolic processes to industrial production (Smedsgaard & Nielsen, 2005). Among the advantages of using metabolites from filamentous fungi are the proliferous secondary metabolism, the concomitant possibility of scaling up the fermentative process, and the fact that, even common species of fungi, like those from *Penicillium* genus, known for their ubiquity, can produce active metabolites (Takahashi & Lucas, 2008). The use of microbial metabolites in foods also reflects the worldwide boom in the preference of consumers for substances extracted from natural sources. Natural additives are usually associated with healthy and good quality products while synthetic substances tend to be labeled as harmful, and, in fact, some of them are responsible for allergic reactions and intolerances, like tartrazine (Yellow Number 5) (Blendford, 1995). Several studies linking the consumption of some artificial colorants and hyperactivity in children were also published (Wrolstad & Culver, 2012).

In some countries like Japan and China, food colorants from natural sources have long been used. Some fungi from *Monascus* genus, producers of colored compounds named azaphilones, are used in fermentation processes to make red yeast rice, as food colorant or food supplement (Patakova, 2013). The azaphilones are a class of compounds containing a pyrano-quinone ring on a highly unsaturated structure and widely accepted as food additives in Eastern countries (Erdoğrul & Azirak, 2004). These compounds have been isolated from the culture of other fungi like *Chaetomium globosum* (Borges et al., 2011) and *Dothideomycetes* sp. (Senadeera, Wiyakrutta, Mahidol, Ruchirawat, & Kittakoop, 2012).

Penicillium sclerotiorum is a mesophyll microorganism, with green color and cotton aspect. Its extracts, obtained with hexane and ethyl acetate, have antimicrobial and anti-HIV activities (Arunpanichlert et al., 2010). It is also reported the production of enzymes of industrial importance by *Penicillium sclerotiorum* (Knob & Carmona, 2009). The predominant chemical constituent in *P. sclerotiorum* is an azaphilone called sclerotiorin (**1**) (Fig. 1), a pigment with strong orange color (MacCurtin & Reilly, 1940). Studies have shown that this compound has some interesting biological

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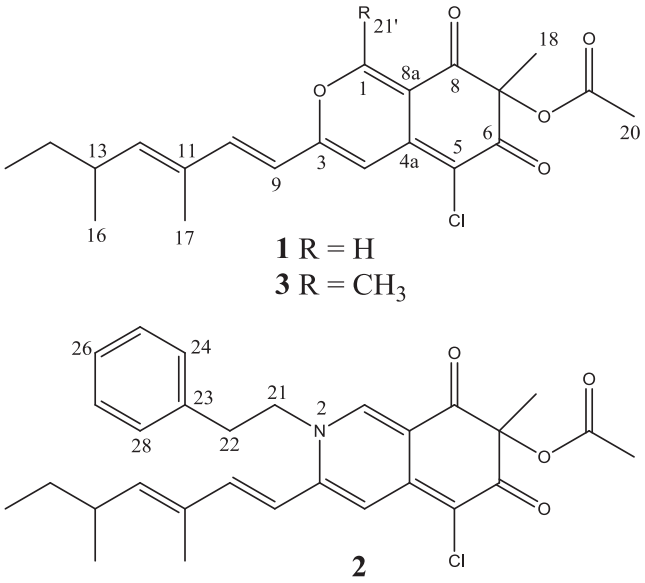

Fig. 1. Molecular structures of sclerotiorin (**1**), *N*-ethylbenzene-sclerotioramine (**2**) and 1-methyl-sclerotiorin (**3**).

activities of interest for pharmaceutical and food industries, as the inhibition of lipoxygenase, which justifies its use as an anti-oxidant (Chidananda & Sattur, 2007), reduction of plasma cholesterol levels (Tomoda et al., 1999) and in the treatment of diabetes (Chidananda, Rao, & Sattur, 2006). It is also reported to induce chlamydospore formation (Weng, Su, Choong, & Lee, 2004). Other uses include inhibition of endothelin receptor (Pairet et al., 1995), inhibition of monoamine oxidase, considerable antibacterial activity (Weng et al., 2004), inhibition activity of HIV protease and invertase (Arunpanichlert et al., 2010), and even antitumor activity (Giridharan, Verekar, Khanna, Mishra, & Deshmukh, 2012).

Safety of sclerotiorin in foods can be presumed since there are reports on its low toxicity as well as its use as food deterioration retarder (Negishi, Matsuo, Miyadera, & Yajima, 2000). In addition, *P. sclerotiorum*, used for producing sclerotiorin, is part of a microorganisms consortium used to produce fermented food in Africa (Amoa-Awua, Frisvad, Sefa-Dedeh, & Jakobsen, 1997).

The large number of biological activities described for sclerotiorin (**1**), as well as its strong color allows foreseeing possibilities for its use in food industry, not only to provide color and antioxidant characteristics in food, but also as nutraceuticals. The use of *Monascus* pigments as nutraceuticals have been well studied and described in the literature (Kim et al., 2007; Kuo, Hou, Wang, Chyau, & Chen, 2009).

Microbial transformation or biotransformation consists in chemical modifications, usually of xenobiotic compounds, catalyzed by whole cells or isolated enzymes (Faber, 2011). Due to its high regio and stereoselectivity, microbial transformations complement organic synthesis and has been widely applied to pharmaceutical and food industry (Rai, 2009, chap. 8), like the conversion of tea catechins to theaflavins (Sharma, Bari, & Singh, 2009), or to obtain aroma compounds from carotenes (Uenojo, Junior, & Pastore, 2007).

Beauveria bassiana has often been employed in biotransformation studies due to its efficient enzyme system, wide acceptability of substrates and its ability to catalyze different types of biotransformation. It has been reported that this fungus is able to biotransform over than 300 different types of substrates. *Beauveria bassiana* is the second microorganism most often used as biocatalyst, behind only *Aspergillus niger*, and its general applications are surpassed only by *A. niger*, *Pseudomonas putida* and *Saccharomyces cerevisiae* (Rai, 2009).

Besides its uses as a biocatalyst, the fungus *B. bassiana* has been utilized as a harvesting natural insecticide in crops used for human consume. Studies concerning this microorganism metabolism, as well its interaction with invertebrates and also mammals, show that *B. bassiana* can be considered safe (Feng, Propawski, & Khachatourians, 1994; Zimmerman, 2007).

This work aims to compare the effectiveness of sclerotiorin's biotransformation by *B. bassiana* using growing cells and resting cells, in order to obtain novel colorants.

2. Materials and methods

2.1. Reagents

The reagents glucose, KH_2PO_4, NaCl and $MgSO_4$ (Synth, Brazil) were of analytical grade. The culture media, bacteriological peptone and yeast extract were from Himedia (India). Silica gel used for column chromatography (230–400 mesh) was purchased from Sigma-Aldrich (USA). The HPLC grade solvents hexane, dichloromethane, ethyl acetate and methanol were purchased from J. T. Baker (USA).

2.2. Instruments

For HPLC analysis, the chromatograms were obtained on a Shimadzu Prominence System (Kyoto, Japan) with two LC-20AT pumps, using reverse polarity C18 column (Supelcosil LC18) and UV detector SPD-20A. ^1H, ^{13}C NMR and bidimensional NMR spectra Heteronuclear two-dimensional single-quantum correlation spectroscopy (HSQC), Heteronuclear multiple-bond correlation spectroscopy (HMBC), Correlation spectroscopy (COSY) and Nuclear Overhauser effect spectroscopy (NOESY) were measured at 300 K on a Bruker AVANCE DRX 400 Spectrometer (Rheinstetten, Germany) equipped with a ^1H-^{13}C 5 mm dual probe. Tetramethylsilane was used as internal reference. Spectra were obtained at 400 or 200 MHz for ^1H and 100 or 50 MHz for ^{13}C. NMR samples were prepared by dissolving the compounds in $CDCl_3$ (Sigma-Aldrich, St. Louis, USA) containing TMS (0.05%). Mass spectrometry analyses were performed on a Micromass Q-TOF Micro spectrometer (Manchester, UK). Elemental composition analysis was performed on a CHN 2400 – Perkin Elmer Elemental Analyzer (Waltham, USA).

2.3. Production of sclerotiorin (*1*)

Spores of *P. sclerotiorum* stored in a 12% v/v glycerol aqueous solution were transferred to 500 mL Erlenmeyer flasks containing 200 mL of sterilized liquid medium 1 (2% glucose, 0.5% bacteriological peptone, 0.1% KH_2PO_4, 0.5% NaCl, 0.05% $MgSO_4{\cdot}7H_2O$, w/v). The flasks were kept under orbital stirring (120 rpm) for 3 days. This culture was transferred into 6 L Erlenmeyer flasks, containing liquid medium 2 (1% glucose, 0.25% bacteriological peptone, 0.05% KH_2PO_4, 0.25% NaCl, 0.025% $MgSO_4{\cdot}7H_2O$) (7 L). The flasks were incubated for 21 days at room temperature without stirring. After this period, mycelia and liquid media were separated by filtration using filter paper and extracted separately to ensure optimal removal of components from both the liquid medium and the mycelium. The solid mycelium was soaked in ethyl acetate while the liquid medium was extracted in a separator funnel (three times). The ethyl acetate extracts were pooled, and the solvent was evaporated. The crude extract (3.07 g) was purified by column chromatography using hexane, dichloromethane, ethyl acetate, and methanol in mixtures of increasing polarities, to obtain 914.2 mg (30% yield) of sclerotiorin (**1**) (dichloromethane:ethyl acetate 95:5) as orange crystals (m.p.

199–202 °C). HRMS: $[M]^+$ *m/z* 391.1370 (calculated for $C_{21}H_{23}ClO_5$, 391.1307). NMR data: see Table 1.

2.4. General biotransformation procedures

Spores of *B. bassiana* stored in a 12% glycerol solution were transferred to 500 mL Erlenmeyer flasks containing 200 mL of sterilized liquid medium 1. The flasks were stirred (120 rpm) on an orbital shaker for 3 days. This culture was used in the subsequent experiments.

Small scale biotransformation experiments were executed in a 500 mL Erlenmeyer flask containing 200 mL of biotransformation medium. Large scale experiments utilized 500 mL Erlenmeyer flasks, totalizing 2 L of culture medium. The extract control was obtained by growing the fungus *B. bassiana* for 14 days and extracting its biomass. A control containing only sclerotiorin in distilled water (or culturing medium) was prepared to detect chemical stability of sclerotiorin during the 14 days os biotransformation.

2.5. Biotransformation with growing cells

This experiment was carried on in small scale. *B. bassiana* culture was transferred to a 500 mL Erlenmeyer flask containing 200 mL of sterilized liquid medium 2. After 3 days, 1 mL of a solution containing sclerotiorin (**1**) in ethyl acetate (20 mg/mL) was added to each flask. The biotransformation took place for 14 days on an orbital shaker (120 rpm) at 25 °C. After this period, medium and mycelia were separated by filtration, and extracted separately, as previously described. The extract obtained (BE1) was analyzed by HPLC.

2.6. Biotransformation with resting cells

B. bassiana culture was inoculated into a 500 mL Erlenmeyer flask containing 200 mL of sterile liquid medium 3 (2% glucose, 1% bacteriological peptone, 0.05% yeast extract) without agitation, for 10 days. The mycelium formed was separated from the broth by filtration under sterile conditions and transferred to Erlenmeyer flasks containing 200 mL of sterile distilled water. Then 1 mL of a solution containing sclerotiorin (**1**) in ethyl acetate (20 mg/mL) was added. Biotransformation took place for 14 days on an orbital shaker (120 rpm) at 25 °C. After this period, mycelia and medium were separated by filtration and both were extracted with ethyl acetate, using the procedure already described. The extract obtained (BE2) was analyzed by HPLC. This experiment was also repeated in large scale. An amount of 200 mg of **1** was added to 2 L of sterile distilled water containing *B. bassiana* mycelia. This resulted, after 14 days of biotransformation followed by work up, in 360.8 mg of dry extract. The product, *N*-ethylbenzene-sclerotioramine (**2**) (27.5 mg, 13.8% yield) was isolated (retention time 18.060 min), after column chromatography, eluted with dichloromethane/ethyl acetate (85:15) (red crystals, m.p. 110–112 °C). HRMS: $[M]^+$ *m/z* 493.2060 (calculated for $C_{29}H_{32}ClNO_4$, 493.2020), C: 68.95%, H: 6.23%, N: 2.80% (calculated for $C_{29}H_{32}ClNO_4$, C: 70.51%, H: 6.53%, N: 2.84%), NMR data: see Table 1.

2.7. Preparation of 1-methyl-sclerotiorin (**3**)

Diazomethane was prepared from Diazald (Sigma-Aldrich, St. Louis, USA) (10.4 g) in diethyl ether (80 mL) on a round bottom flask coupled with a side condenser containing an addition funnel

Table 1
NMR chemical shifts of sclerotiorin (**1**), *N*-ethylbenzene-sclerotioramine (**2**) and 1-methyl-sclerotiorin (**3**).

Position	Sclerotiorin (**1**)			*N*-Ethylbenzene-sclerotioramine (**2**)			1-Methyl-sclerotiorin (**3**)		
	δ_C (ppm)	δ_H (ppm)	HMBC	δ_C (ppm)	δ_H (ppm)	HMBC	δ_C (ppm)	δ_H (ppm)	HMBC
1	152.6	7.93 (s^1)	C-3, C-4a, C-8a	140.9	7.54 (s)	C-3, C-8, C-21	166.9		
3	158.1			148.0			156.7		
4	106.4	6.65 (s)	C-3, C-5, C8a	111.6	6.97 (s)	C-4a	106.5	6.72 (s)	C-3, C-8a, C-9
4a	138.7			102.4			140.5		
5	110.8			144.3			109.9		
6	186.0			184.4			185.7		
7	84.6			84.8			85.3		
8	191.8			193.8			192.9		
8a	114.6			114.6			110.7		
9	115.7	6.08 (d^1); *J* 16 MHz	C-3, C11	114.6	5.98 (d); *J* 15.6 MHz	C-4, C-11	115.7	6.07 (d); *J* 15.8 MHz	C-3, C-4, C-11
10	142.9	7.06 (d); *J* 16 MHz	C-3, C-17	145.0	6.90 (d); *J* 15.6 MHz	C-12, C-17	142.3	7.06 (d); *J* 15.8 MHz	C-3, C-12, C-17
11	132.0			131.5			132.0		
12	148.8	5.70 (d); *J* 10 MHz	C-10, C-17	147.8	5.68 (d); *J* 10 MHz	C-10, C-14, C-17	148.4	5.72 (d); *J* 9.8 MHz	C-10, C-13, C-14, C-16, C-17
13	35.1	2.49 (m^1)		35.1	2.48 (m)		35.1	2.50 (m)	
14	30.1	1.37 (m)		30.0	1.40 (m)		30.1	1.36 (m)	
15	11.9	0.87 (t^1); *J* 7.6 MHz	C-13, C-14	12.0	0.89 (t); *J* 7.2 MHz	C-13, C-14	12.0	0.88 (t); *J* 7.4 MHz	C-13, C-14
16	20.2	1.01 (d); *J* 6.8 MHz	C-12, C-13, C-14	20.2	1.03 (d); *J* 6.4 MHz	C-12, C-13, C-14	20.3	1.03 (d); *J* 6.6 MHz	C-12, C-13, C-14
17	12.4	1.85 (s)	C-10, C-11, C-12	12.6	1.81 (s)	C-11	12.4	1.86 (s)	C-10, C-11, C-12
18	22.5	1.57 (s)	C-6, C-7, C-8	23.2	1.52 (s)	C-6, C-7, C-8	22.9	1.57 (s)	C-6, C-7, C-8
19	170.1			170.0			169.8		
20	20.1	2.17 (s)	C-19	20.3	2.16 (s)	C-19	20.2	2.19 (s)	C-19
21′							20.2	2.65 (s)	C-1, C-8a
21				55.4	4.05 (m)	C-1, C-3, C-23			
22				36.6	3.03 (m)	C-23, C-24,			
23				135.8					
24				129.3	7.26 (s)	C-22, C-26			
25				128.7	7.10 (d); *J* 6.8 MHz				
26				127.7	7.31 (m)	C-23, C-28			
27				128.7	7.10 (d); *J* 6.8 MHz				
28				129.3	7.26 (s)	C-22, C-26			

s: singlet, d: doublet, t: triplet, m: multiplet.

on the top. This solution was drop wise added to a flask containing 100 mL of a 10% sodium hydroxide aqueous solution and 200 mL of ethanol and the mixture was heated at low temperature (60 °C). Ethereal diazomethane was condensed and added to a flask containing 900 mg of sclerotiorin (**1**) dissolved in diethyl ether until a yellow color remained in the flask indicating diazomethane excess. After 10 min, the solvent was evaporated at reduced pressure. The reaction product was purified by flash column chromatography using 100% dichloromethane as eluent to obtain 1-methyl-sclerotiorin (**3**) (31% yield) (orange crystals, m.p. 177–179 °C), HRMS: $[M]^+$ *m/z* 404.2026 (calculated for $C_{21}H_{23}ClO_5$, 404.1391). NMR data: see Table 1.

2.8. Biotransformation of 1-methyl-sclerotiorin (*3*)

The biotransformation of **3** was carried out using resting cells following the procedure already described for the biotransformation of sclerotiorin (**1**). The extract obtained (BM2) was analyzed by HPLC.

2.9. HPLC analysis

Aliquots of the biotransformation extracts were dissolved in methanol (1 mg/mL) and filtered through a membrane (Millex LCR 0.45 μm PTFE). The eluents used were: acetonitrile (solvent A) and water (solvent B) plus 0.05% of formic acid. The gradient used was solvent A: solvent B (1:1) to 100% of solvent A in 30 min at 1.0 mL/min, 100% of solvent A for 10 min at 1.0 mL/min. The injected volume was 20 μL. The UV detector was set to 470 nm (wave length able to detect compounds in the range of red and orange color) and 530 nm (detection of red compounds only).

3. Results

3.1. Substrates preparation

Scleotiorin (**1**) produced by *P. sclerotiorum* after 21 days of fermentation was purified by column chromatography. The mass of this metabolite obtained after purification represented 30% w/w of the whole fungal extract.

Analysis of ^1H and ^{13}C NMR in addition to the 2D spectra were able to confirm the structure of the isolated product as sclerotiorin (**1**). All the signals from the pyrone-quinone bicyclic ring structure were present. There were found correlations in HMBC contour map between atoms of the two rings, such as the correlations between C-5 and H-4, and H-4 and C8a. Presence of an aliphatic side chain at C-3 was corroborated by the HMBC correlations between H-9 and H-10 to C-3. The chemical shifts of **1** are similar to those described in the literature for this molecule (Chidananda & Sattur, 2007). Full NMR data are shown in Table 1.

3.2. Biotransformation of sclerotiorin

The chromatogram of the extract control showed no peaks, neither at 470 nor at 530 nm, showing that the fungus *B. bassiana*, used as the biotransformation agent, produced no colored metabolites absorbing at those wavelengths. The biotransformation of sclerotiorin with growing cells in small scale took place for 14 days. The extract obtained (BE1) was analyzed by HPLC using the UV detector at 470 and 530 nm. A peak corresponding to sclerotiorin was detected by HPLC at 470 nm with retention time of 19.780 min. At 530 nm, sclerotiorin, which has orange color, cannot be detected. Besides sclerotiorin, no other peaks were observed in the chromatogram of extract BE1 (Fig. 2), showing that this process was not efficient for production of colored sclerotiorin derivatives.

For this reason, it was performed a biotransformation using resting cells in which the mycelium of *B. bassiana* was grown, filtered and transferred to Erlenmeyer flasks containing only sterile distilled water and sclerotiorin (**1**). The extract obtained (BE2) was also analyzed by HPLC (Fig. 2), showing new peaks at both 470 and 530 nm. This indicates the presence of biotransformation products that, differently from sclerotiorin, have red color. Analysis of the control experiments showed absence of novel compounds, proofing that the products formation was not related to the chemicals present in the culturing medium. After the biotransformation, 138.1 mg of sclerotiorin (69.1%) was recovery unaltered from the experiment.

3.3. Structural elucidation of the biotransformation product

After identifying formation of biotransformation products from sclerotiorin (**1**) using resting cells, this experiment was repeated, in large scale, and one product was isolated. The other substances identified by HPLC were not isolated in amount enough for spectroscopic identification. The HRMS of the biotransformation product gave an ion peak at *m/z* 493.2060, consistent with the molecular formula $C_{29}H_{32}NO_4Cl$. Elemental analysis confirmed the presence of a nitrogen atom.

Analysis of ^{13}C NMR spectra of **2** showed that all the signals present in the spectrum of the starting material were also present in the spectrum of the biotransformation product, besides eight new signals (C-21 to C-28), typical of an ethylbenzene system. This assignment was corroborated by sub spectrum DEPT, which indicated that two of the new carbons were methylene and five were methine. Analysis of the COSY contour map showed correlations among the hydrogens of the aromatic ring. The quaternary aromatic carbon (C-23) presented J^2 correlations with methine hydrogens H-24 and H-28 in the HMBC spectrum and with methylene hydrogens of C-22. There were also observed correlations in the HMBC contour map between the two methylene groups (C-21 and C-22). The chemical shifts indicated that this group was inserted in the original molecule through position 2, where the oxygen atom was exchange by a nitrogen atom. In the HMBC contour map it was noted that the H-21 from the ethylbenzene substituent has J^3 correlations with C-1 and C-3, corroborating the assignment of the nitrogen atom. Other correlations presented in HMBC contour map are represented in Fig. 3.

3.4. Synthesis of 1-methyl-sclerotiorin

Sclerotiorin (**1**) was used to prepare its 1-methyl derivative (**3**, Fig. 1) by reaction with diazomethane, a very efficient methylating agent. Compound **3** was purified by column chromatography using the same eluent gradient described in item 2.3. In the fraction eluted with 100% dichloromethane, the product was obtained (276.9 mg; 31% yield). Analysis of ^1H and ^{13}C NMR of **3** showed great similarity with the spectra of the starting material, except for the absence of the singlet at 7.93 ppm (H-1). Methylation of C-1 was proved by the singlet detected at 2.65 ppm (3H). The HSQC contour map spectrum showed that this new signal was correlated to the methyl carbon at δ_C 20.2 ppm. The hydrogen atoms of this new methyl group showed J^2 correlations with C-1 and J^3 correlations with C-8a in the HMBC spectrum. This new product, identified as 1-methyl-sclerotiorin (**3**) is herein described in literature for the first time. The full NMR data assignments are shown in Table 1.

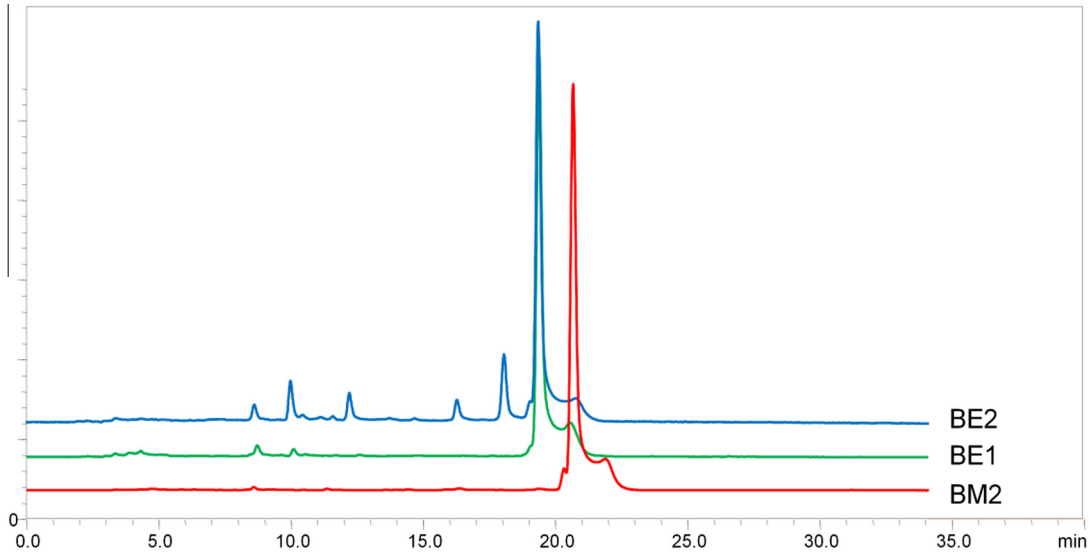

Fig. 2. HPLC chromatogram (470 nm) of extracts BE1 (biotransformation of sclerotiorin using whole cells), BE2 (biotransformation of sclerotiorin using resting cells) and BM2 (biotransformation of 1-methyl-sclerotiorin using resting cells).

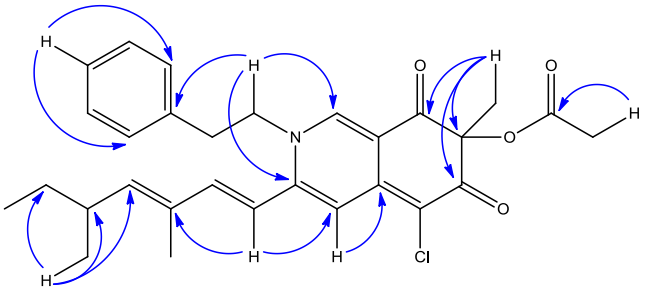

Fig. 3. Correlations presented in HMBC contour map to *N*-ethylbenzene sclerotioramine (**2**).

3.5. Biotransformation of 1-methyl-sclerotiorin

Biotransformation of 1-methyl-sclerotiorin by *B. bassiana* was carried out using resting cells in small scale, since it was proved to be efficient towards sclerotiorin. The biotransformation extract obtained under these conditions was analyzed by HPLC, using UV detector (470 and 530 nm). The product 1-methyl-sclerotiorin presented a retention time of 20.668 min. The chromatogram of its biotransformation extract (BM2) (Fig. 2) did not present any new peak at 470 or 530 nm.

4. Discussion

Penicillium pigments, although produced in high yields, are still not fully investigated, even in the Eastern countries, where there is a traditional use of fungal metabolites in foods. The fungi of genus *Penicillium* have the advantage of being ubiquitous, present in many environments, generally not causing complications for human health.

Biotransformation is an efficient method to carry out chemical modifications in organic compounds. Fungal enzymatic metabolism makes possible to obtain products that would be difficult to achieve by other conventional methods of synthesis (Takahashi, Gomes, Lyra, Santos, & Martins, 2014). Sclerotiorin (**1**) is a good target for obtaining molecules with improved pharmacological or industrial properties. Biotransformation experiments with this molecule seems never been reported before.

The fungus *P. sclerotiorum* produced the metabolite sclerotiorin with high yield (30% w/w of crude extract).

In this study, the biotransformation of sclerotiorin by *B. bassiana* was evaluated using whole cells and resting cells. The main difference between these two approaches is that, using the first methodology, the substrate is added while the cells are still growing in the liquid medium, and, in the latter, all nutrients are removed from the biotransformation media before feeding the substrate to the fungus. When the biotransformation was carried out using resting cells, the process was more efficient probably because removal of the nutrients from the medium forced the microorganism to use sclerotiorin in its metabolism. *N*-Ethylbenzene-sclerotioramine was obtained with 13.8% yield. This is a good initial yield for a biotransformation experiment considering that biotransformation yields rarely exceed 10% prior to optimization steps (Fu et al., 2011).

The first change noticed in the product biotransformed by *B. bassiana* was the change of color from dark orange to red. This indicated that, at some level, the biotransformation modified sclerotiorin's chromophore. This is a very interesting accomplishment since obtaining derivatives of different colors increases the range of azaphilones applications in pharmaceutical and food industries.

The structural elucidation of *N*-ethylbenzene-sclerotioramine showed the formation of a nitrogenated heterocycle which was confirmed by NMR spectroscopy, elemental analysis and mass spectrometry. Tomoda et al. (1999) described a synthetic reaction in which primary amines are capable of reacting with azaphilones in strongly alkaline medium. Latter, the mechanism of this reaction was described by Wei and Yao, showing that the oxygen-bearing heterocyclic ring is opened due to a nucleophilic attack by the amine. The oxygen atom is lost as a water molecule and a nitrogenated heterocycle is formed (Fig. 4) (Wei & Yao, 2005). Mapari and collaborators showed that, among azaphilones, generally those containing a nitrogen atom in the typical bicyclic ring pyronequinone, tend to have reddish color (Mapari, Thrane, & Meyer, 2010).

Atom exchange reactions are commonly described in the literature. Many fungi are reported as being capable of replacing oxygen atom by nitrogen in biological molecules. Enzymes such as transaminases present in fungi and other microorganisms are described as capable of performing this atom exchange (Klatte & Wendisch, 2014; Mutti, Fuchs, Pressnitz, Sattler, & Kroutil, 2011).

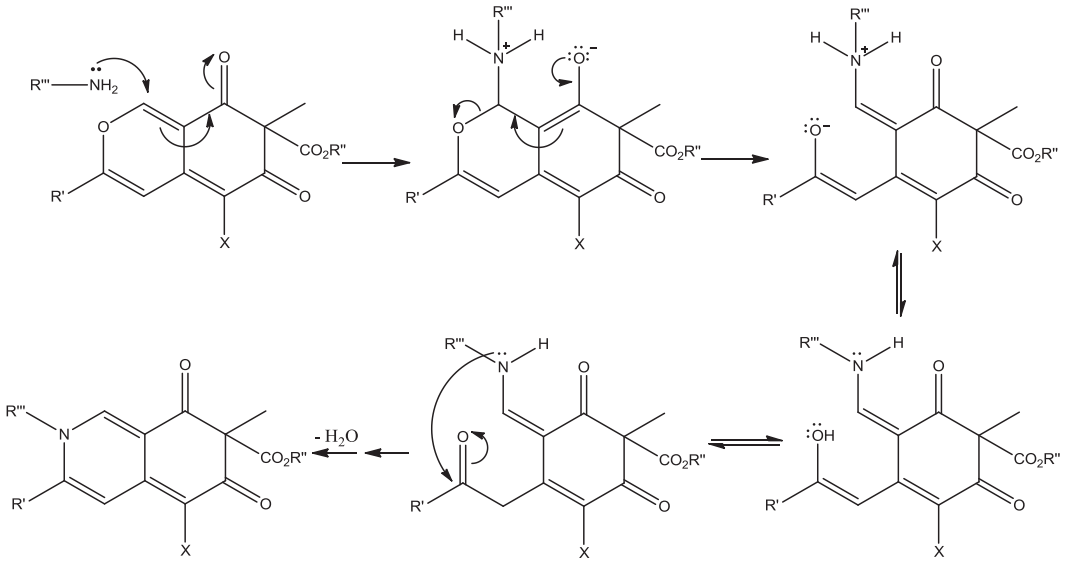

Fig. 4. Heteroatom exchange mechanism proposed by Wei and Yao. Adapted from Wei and Yao (2005).

Currently, laccases-producing fungi have already been used to perform amines condensation reactions to create bioactive molecules (Mikolasch et al., 2008; Niedermeyer & Lalk, 2007). The fungus *B. bassiana* is widely found in the literature as catalyst for many reactions. Studies on the biosynthetic routes to production of metabolites by this species show that enzyme systems able to perform heterocyclic ring opening is found in this microorganism (Halo et al., 2008), corroborating the results herein described. Moreover, compound 2 has already been described as a secondary metabolite isolated from the fungus *Diaporthe* sp. using the same biosynthetic route proposed in the present work starting from sclerotiorin (Zang et al., 2012).

According to the mechanism proposed by Wei and Yao (2005), an initial attack at carbon 1 is essential for the switching of heteroatoms and formation of the products like compound **2**. To explore this fact, 1-methyl-sclerotiorin (**3**) was synthesized. The methyl group was inserted at position 1, next to the oxygen heteroatom. Once carbon-1 was blocked, biotransformation no longer occurred, as evidenced by the chromatogram of 1-methyl-sclerotiorin biotransformation. Since bioreactions involve participation of enzymes, most of them regio and stereospecific, it is likely that chemical modifications are very sensitive to changes in the binding site of the enzyme.

New methodologies for the synthesis of new food-friendly red colorants have great importance due to some factors such as the limited availability of red pigments for use in food industry, to the prohibition of Red No. 2 and the continued scrutiny of Red No. 40 and Red No. 3 (Hallagan, 1991). Interestingly, studies with *Monascus* pigments showed that some red derivatives, produced from yellow azaphilones by synthesis using strong base (sodium hydroxide) and reflux, were more stable to the light in aqueous solution in comparison to orange pigments naturally produced by the fungus. (Wong & Koehler, 1983).

5. Conclusion

Sclerotiorin was produced on a good yield from *P. sclerotiorum*, and purified to be used in the biotransformation. Resting cells methodology was more useful than the use of whole cells for products formation. The major product of biotransformation was isolated and fully identified by spectroscopic methods as *N*-ethylbenzene-sclerotioramine, a derivative resulting from heteroatom replacement by *B. bassiana*. Heteroatom change also resulted in color modification, since sclerotiorin is yellow and its nitrogenated product has a strong red color. Sclerotiorin, when methylated at C-1, was not converted in biotransformation products, showing that the heteroatom replacement mechanism is dependent of the hydrogen atom located at C-1. The isolation of a red azaphilone indicated that biotransformation of sclerotiorin by the fungus *B. bassiana* can be an interesting green tool to produce reddish derivatives to be used as food colorants.

Use of sclerotiorin and its derivatives on an industrial scale should be taken into consideration, since it showed high initial production yields by the fungus *P. sclerotiorum* and encouraging yield by biotransformation with *B. bassiana*. Preparation of new azaphilones is of great importance since they are already being used in oriental foods, and are structurally different of industrial food colorants related to allergic processes, such as tartrazine. This kind of colorants is welcome by consumers nowadays, since they are extracted from natural sources, and, therefore, they are more aligned to the modern consumers' preference. Although conversion of orange azaphilones in red derivatives is already described in the literature, the process herein described, via biotransformations, was accomplished using very mild conditions, which is a great advance for a greener production of red azaphilones.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.04.057.

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