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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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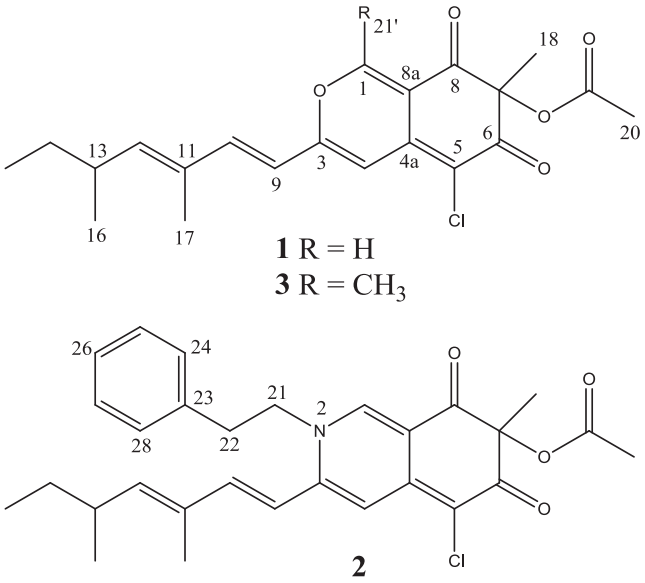

Fig. 1. Molecular structures of sclerotiorin (**1**), *N*-ethylbenzene-sclerotioramine (**2**) and 1-methyl-sclerotiorin (**3**).

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.