Tropical Journal of Pharmaceutical Research August 2010; 9 (4): 365-371
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Research Article

Improved Production of Pharmacologically-active Sclerotiorin by *Penicillium sclerotiorum*

Esther MF Lucas[1,2], Yuri Machado[1], Alexandre A Ferreira[1], Luiza MP Dolabella[1] and Jacqueline A Takahashi[1]*

[1]Departamento de Química, ICEx, Universidade Federal de Minas Gerais, Av. Antonio Carlos, 6627, CEP 31270-901, Belo Horizonte, MG, [2]Departamento de Química, Universidade Federal de Lavras, Campus Universitário, Centro, CEP 37200-000, Lavras MG, Brazil

Abstract

Purpose: *The aim of this work was to study the optimum conditions for the production of sclerotiorin, a highly active secondary metabolite of Penicillium sclerotiorum under different cultural media.*
Methods: *A Brazilian strain of P. sclerotiorum was grown under different culture conditions in two liquid media (malt and a dextrose-peptone salt medium supplemented with sodium chloride) and in solid state fermentation in rice. Sclerotiorin production was monitored by high performance liquid chromatography (HPLC).*
Results: *Quantitative analysis of sclerotiorin content by HPLC indicated that sclerotiorin production reached the highest level (up to 313 $\pm$ 10 mg.L^{-1}) in the dextrose-based medium after 10 days of fermentation. Rice and malt broth showed lower production levels.*
Conclusion: *Enhanced production of P. sclerotiorin for pharmaceutical development can be achieved using dextrose-based cultures.*

Keywords: *Penicillium sclerotiorum, Sclerotiorin,Yield improvement, HPLC, Pharmaceutical industry*

Received: 1 October 2009 *Revised accepted: 8 May 2010*

Corresponding author:* ***E-mail:* *jat@qui.ufmg.br;* ***Tel:*** *+5531 34095754;* ***Fax:*** *+5531 34095700;*

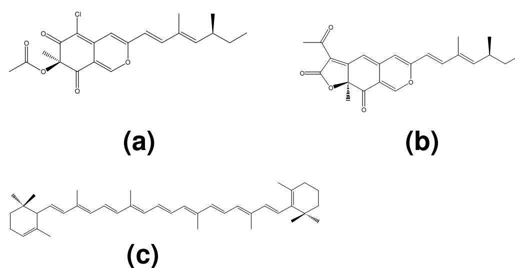
INTRODUCTION

Bioactive secondary metabolites possessing interesting chemical structures have been widely described but further development of such compounds as new drugs by the pharmaceutical industry are not frequently accomplished, especially due to the low yield obtained from many of the naturally occurring molecules. Therefore, a limiting step at industry-level pharmaceutical production of bioactive natural compounds is reliance on their large scale availability. This limitation can be overcome, when possible, by organic synthesis. However, a synthetic approach may be not feasible due to low final yields or to the structural features of the active molecule. Alternatively, an optimized extraction of the natural product from a natural source can be targeted, and for plant metabolites, a good alternative relies on the use of cell culture. For microbial metabolites, bench culturing conditions can be determined and scaled up in order to reach adequate levels of metabolite yield [1].

Penicillium is a very important genus for the production of valuable industrial products such as penicillin and statins. The fungus, *Penicillium sclerotiorum* (synonym *Penicillium multicolor*), produces a class of secondary biological active metabolites named azaphilones [2-5]. The chlorinated azaphilone sclerotiorin (see Fig 1(a)) was the first secondary metabolite isolated from this microorganism [6]. There are biological activities related to sclerotiorin (Fig (1a)) such as endothelin receptor binding [7] and lipase inhibition, leading to its use in Japan to prepare anti-acne creams and biscuits [8]. Sclerotiorin also inhibits aldose reductase, an enzyme associated with complications such as cataract, neuropathy and nephropathy in diabetic patients [9]. It has also been reported to be active against the bacteria *Micrococcus luteus*, *Bacillus cereus, Bacillus subtilis, Klebisiella pneumoniae, Escherichia coli, Salmonella typhimurium, Lysteria monocytogenes* [9] as well as *Streptomyces pyogenes, Staphylococcus aureus* and the yeast *Candida albicans* [10]. Besides the importance of the biological properties presented by this metabolite, its strong orange color makes sclerotiorin also makes it an interesting colouring additive in food and cosmetic products.

Studies on the biosynthesis of sclerotiorin have demonstrated that the skeleton of this substance is formed by the linkage of nine molecules of acetate (from acetic acid) and three molecules of formate (from formic acid), following an acetate-malonate pathway, and involving the condensation of two poli-β-ketide chains in which the terminal malonate molecules are decarboxilated to the acetate before being incorporated to the chain [11]. Nutritional conditions can affect considerably secondary metabolite production and it is known that the absence of a chlorine donor in the culture medium suppresses sclerotiorin production [12]. When *P. sclerotiorum* was grown on Czapek-Dox media, sclerotiorin was reported to be the major metabolite, but in Raulin-Thom media, rotiorin (Fig 1(b)) was the major metabolite. Azaphilones production by *P. sclerotiorum* has been related to high levels of dextrose in media at pH 5.9 – 6.0 [6], while at pH 2.5, the metabolites produced were shown to be α, β and γ-carotenes (see Fig 1(c) – (e)) [13].

Aiming at setting an optimized protocol for large scale production of sclerotiorin, a Brazilian strain of *Penicillium sclerotiorum* was cultivated under different conditions such as duration of fermentation and culture medium composition. Sclerotiorin production was quantified by HPLC.



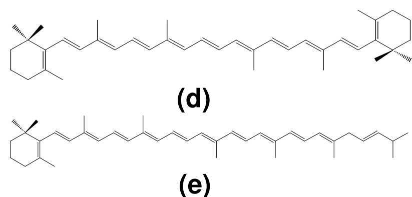


Figure 1: Structures of *P. sclerotiorum* metabolites

EXPERIMENTAL

Chemicals, standard and media components

Culture medium components were purchased from Biobrás (Montes Claros, Brazil), Merck (Darmstadt, Germany) and DIFCO Laboratories (Michigan, EUA). The rice used was a Brazilian commercial brand - Tio João, 25JAN071A. Solvents and TLC eluents were purchased from Grupo Química (São Paulo, Brazil) while HPLC eluents were obtained from Merck. Sclerotiorin (structure (a) in Fig 1) used to derive the calibration curve was obtained (by recrystalisation in ethanol) from the organic layer solids of a 15-day old culture of *Penicillium sclerotiorum*. It was identified by melting point and by molecular spectroscopy in the infrared region as well as by nuclear magnetic resonance spectroscopy.

Microorganism

P. sclerotiorum LAB18 was isolated from a soil sample collected in Minas Gerais, Brazil [14] by Biotechnology and Bioassays Laboratory (LaBB) Collection (Chemistry Department, ICEx, UFMG). The fungal strain was maintained on potato dextrose agar (PDA) and refrigerated at 7 ^{o}C.

Culture media preparation

Three liquid media were used in this screening, namely, rice, malt broth and a medium of complex molecular composition. Rice medium (RM) was prepared by adding 11 g of rice to 250 mL of distilled water in ten conical flasks that were left to stand for 24 h. Malt broth medium (MB) was prepared by adding 40 g of malt to 2 L of distilled water and 200 mL of this solution was transferred to each of ten 500 mL conical flasks. Complex medium (CM) was prepared by adding dextrose (40 g), peptone (10 g), KH_2PO_4 (2 g), $MgSO_4.7H_2O$ (1 g) and NaCl (10 g) to 2 L of distilled water. An aliquot of 200 mL of this solution was transferred to each of the ten 500 mL conical flasks. Media pH varied between 5.6 and 6. All flasks were sterilized in an autoclave for 15 min at 121 ^{o}C.

Inoculation of *P. sclerotiorum*

In a test tube containing *P. sclerotiorum* in PDA medium, 40 mL of distilled sterilized water was added. Superficial fungal mycelium was scraped off and the suspension obtained was dispersed using a vortex apparatus. An aliquot of 1 mL of this suspension was transferred to each of the flasks containing the three different culture media. This operation was performed in aseptic conditions in a flow cabinet. The flasks were kept at room temperature (25 ± 2 ^{o}C).

Preparation of extracts

After 48 h, 100 mL of ethyl acetate was added to one flask of each culture medium to stop fungal growth. This system was left to stand for three days, filtered and transferred to a separating funnel. The aqueous phase was successively extracted three times with 100 mL of ethyl acetate, producing a solution which was evaporated to dryness by vacuum distillation. The extracts obtained were dissolved in acetonitrile (1mL), vacuum-filtered, and then evaporated by air drying at room temperature.

Thin layer chromatography (TLC)

All extracts, dissolved in ethyl acetate, were evaluated by TLC with sclerotiorin (structure (a)) as standard. Elution was carried out with hexane:ethyl acetate 2:1 (v/v). The TLC

adsorbent used was 60 G silica gel (Merck, article 7731), with a layer thickness of 0.25 mm on glass and activated at 100 °C for 30 min. TLC plates were viewed on UV-Vis., iodine fumes and Godin solution (comprised of 1 % vanillin in methanol and 3 % perchloric acid in water, 1:1).

HPLC analysis

HPLC analysis was performed on a Shimadzu equipment, series LC 10A, using a Shimadzu Shim-pack C18 column (250 mm x 4.6; 5µm) and a photo array diode (PAD) detector, model SPD 10 A vp. From each of the extracts obtained, samples (1 mg/mL) were prepared by dissolving in acetonitrile, and 50 µL of this solution was injected into the HPLC in triplicate. The mobile phases were aqueous phosphoric acid solution at a concentration of 0.02 mol/L (Pump A) and methanol (Pump B) at the following gradient flow rates: 0 to 25 min – 75 % of methanol; 25 to 27 min - 75 to 100 % of methanol; 27 to 30 min – 100 % of methanol; 30 to 32 min - 100 to 75 % of methanol (40 min total run time). The UV detector was set to 370 nm and the area corresponding to sclerotiorin peaks was registered. Recrystallized sclerotiorin used as standard was also spectroscopically analyzed.

Conversion of area values to concentration values

In order to convert areas to concentration values, an analytical curve was obtained for sclerotiorin in acetonitrile (1 mg/mL). From this solution, 0.5 and 0.4 mL were taken separately to prepare solutions (1 mL each) at concentrations of 0.25 and 0.2 mg/mL, respectively. Solution of 0.25 mg/mL was serially diluted to solutions of 0.13; 0.06; 0.03; 0.02 and 0.01 mg/mL, respectively. Fifty microlitres of each solution were injected in the HPLC apparatus, in triplicate, under the same conditions used to analyze the extracts. The arithmetic means of each concentration was taken. The experimental values obtained were computed by minimum square method using MatLab Mathworks® software to generate the line that best approached experimental values (R^2 = 0.998). The line inclination was determined (1.36 x 10^8 cm^2.mL/mg) and this value was the arithmetic mean of the three values of sclerotiorin peak areas obtained for each extract, was used to give their corresponding concentrations.

Chloride content determination

Mohr method [15] was used to quantify chloride contents in all the culture media (rice, malt broth and peptone). Ten grams of rice were pulverized in a blender for 1 min and transferred to a conical flask containing 40 mL of distilled water. The flask was then heated in a microwave for 3 cycles of 20 s each. The resulting material was filtered over a cloth and the residue was washed with water to obtain a final filtrate volume of 40 mL. The solution was buffered at pH 8.3 using 0.7g of sodium bicarbonate; the experiment was run in triplicate. For chloride determination in malt broth, 300 mg of powdered malt broth, in triplicate, was transferred to 125 mL conical flask containing 40 mL of distilled water and 0.7 g of sodium bicarbonate. The solution was sonicated for 10 min. Complex medium was comprised was chloride-free ingredients apart from peptone and sodium chloride. Therefore, only peptone was screened for its chloride contents, and the same procedure described for malt broth was followed. Next, the samples were directly titrated with standard 0.0397 ± 0.0001 mol/L $AgNO_3$ using 5 mL of a 5 %m/V solution of potassium chromate as end point visual indicator.

Statistical analysis

All experiments were run in triplicate and differences in sclerotiorin concentration data were considered significant at 95 % confidence interval (CI) using Microsoft Excel 2007.

Table 1: Concentration of sclerotiorin (mg/L) in extracts obtained by varying the culture time and nutrition source in *Penicicillium sclerotiorum* cultures

Culture time (days)	Concentration (mean ± SD, *n*=3) of sclerotiorin (mg/L) in extracts		
	Complex	Malt	Rice
2	0	0	0
4	116 ± 1	127 ± 2	0
6	213 ± 4	145 ± 10	0
8	213 ± 6	160 ± 1	125 ± 3
10	313 ± 4	184 ± 4	72 ± 2
12	304 ± 3	203 ± 6	166 ± 3
14	281 ± 3	202 ± 3	157 ± 1
16	262 ± 4	185 ± 2	143 ± 5
18	262 ± 3	200 ± 3	149 ± 1
20	270 ± 11	128 ± 1	82 ± 1

Table 2: Chloride content of the media used and of peptone as determined by Mohr method [15]

Medium/medium component	Chloride level (mean ± SD, *n*=3) (%)
Rice	0.08 ± 0.005
Malt broth	0.24 ± 0.02
Peptone	2.21 ± 0.02
Complex medium (Peptone + sodium chloride)	18.43 ± 0.01

RESULTS

A total of three different media culture were used to achieve optimum production of sclerotiorin. *P. sclerotiorum* presented good growth in all tested media, with abundant mycelial growth, and strong orange color disseminated in the medium that was associated with the production of azaphilones. TLC analysis after 4 days showed that all the extracts prepared in dextrose and malt media had the same TLC profile, consisting of six predominant spots of close polarities. Extracts from the growth of *P. sclerotiorum* in rice showed, initially, a simpler TLC profile, but after 6th day of growth, the same eight-spot profile presented by dextrose and malt extracts was observed.

Sclerotiorin concentration observed for each extract, at 95 % CI, is presented in Table 1. None of the media enabled sclerotiorin production before 4 days of *P. sclerotiorum* cultivation. Maximum release of sclerotiorin in salt-supplemented and malt media occurred on the 10th and 12th day of growth, respectively, corresponding to 313 and 203 mg/L, respectively. The growth in rice led to maximum release on the 12th day of 166 mg/L.

Chloride content was determined for all the media used since sclerotiorin is a chlorinated compound. Rice presented the lowest chloride content (0.08 ± 0.02 %); it was also low in malt broth (0.24 ± 0.06 %). For dextrose-based medium, besides the chloride present in peptone (2.21 ± 0.06 %), an elevated amount of sodium chloride was added (16.22 %w/w) to reach a total chloride level of 18.43 ± 0.03 % (see Table 2).

DISCUSSION

Solid state fermentation (SSF), a useful tool for optimizing enzyme production by fungi as well as by submerged cultures, was used for sclerotiorin yield optimization. Rice was chosen because of its wide use for growing filamentous fungi with excellent outcomes [16]. Rice is an excellent substrate for SSF although for the majority of supports used for SSF, this kind of support involves some problems for scaling up [17].Malt extract, a very common substrate, was selected in order to achieve optimal mycelial growth [18]. The third medium entailed using a richer medium containing generous amounts of dextrose and peptone, an approach which is able to promote improved mycelial growth [19]. Complex media have been regarded as successful choices in real industrial optimization approaches [20]. For the third medium, supplementation with KH_2PO_4 (2g), $MgSO_4.7H_2O$ was accomplished as well as addition of NaCl with the objective of raising sclerotiorin biosynthesis, since addition of substances useful for biosynthesis is a

frequently used alternative approach for yield improvement [2].

The delay in producing the monitored metabolite in rice by *P. sclerotiorum* may be related to the source of nutrients available in this grain since complex medium was supplemented with a monomeric carbohydrate (dextrose) as the major carbohydrate source while in rice, the carbohydrate source was starch, a polymer that must be degraded prior to the release of monomers used in fungal metabolism. Delay in metabolite production in rice medium can also be related to its water content, since it was the least hydrated of the media used in the tests. However, free water content is not considered an extremely restrictive condition for fungal growth by some authors [17]. Overall, no qualitative variation of metabolites was observed by TLC after the 8th day, despite the different sources of nutrients available in dextrose, malt and rice media.

It was observed that sodium chloride-supplemented medium achieved higher production of sclerotiorin with high yields achieved within ten days of growth (313 mg/L). This is an indication that sclerotiorin production is directly associated with the availability of nutrients in the medium. The higher availability of chloride in the dextrose based medium (Table 2) is also consistent with the higher amounts of sclerotorin produced in this medium.

CONCLUSION

In this study, the growth of *P. sclerotiorum* in three different media over period of 20 days showed no apparent difference in terms of the qualitative production of secondary metabolites. However, production of the bioactive metabolite, sclerotiorin, was best accomplished in a medium containing dextrose, peptone, and mineral salts with sodium chloride supplementation. Yield of sclerotiorin in the supplemented medium reached 313 $\pm$ 10 mg.L^{-1}, which was 54.1 % higher than that obtained for malt broth under optimum conditions. This is an interesting initial result as the yield can be considered high and further improvement is still possible. This is particularly important since a leading marketed drug, mevastatin, was reported to have an initial production yield of only 40 mg.L^{-1} from *Penicillium citrinum*, and reaching levels of up to 5g L^{-1} upon optimization [21].

ACKNOWLEDGMENT

This work was supported by International Foundation for Science (IFS – F/3564-2), Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG – CEX APQ-6629-5.02/07 and CEX APQ-5237-5.02/07), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – 307846/2006-7). We thank Paula Pereira Dolabella for assistance with vetting the English language used.

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