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Penicillium sclerotiorum Biomass as a Potential Food Product

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Single cell protein is a biotechnological product made from microorganisms grown under suitable condition for biomass production. A limited number of food products based on single cell protein is available in global market and the most noted example in terms of tangible profits is the mycoprotein Quorn, produced by Marlow Foods from *Fusarium venenatum* mycelium. Fungi, in general, are good raw material for food protein production once they grow in a diversity of affordable sustainable substrates as agricultural straw and bagasse, yielding a nutritionally complete biomass along with other metabolites. Therefore, fungi are important cell factories for food industry. Brazil is one of the world's most biodiverse countries regarding to plants, animals and also fungi, from which innumerable new biotechnological applications can be developed. In the present work *Penicillium sclerotiorum* was cultivated under varied glucose, peptone and sodium chloride concentrations. Biomass yields ranged between 1.89 g and 7.80 g, being greater in culture media with higher glucose contents. Ash levels ranged from 3.55% to 18.11%, on a dry matter basis (d.m.), with macro and micronutrients ranging from 11.9 to 137.5 mg/100 g for Ca, 87.2 to 154.2 mg/100 g for Mg, 0.9 to 3.0 mg/100 g for Zn (no significant difference), 0.6 to 1.7 mg/100 g for Fe (no significant difference), based on d.m. Protein levels ranged from 21.56% to 41.88% based on d.m. Fatty acid profiles revealed the presence of essential fatty acids linoleic and linolenic, the former in greater amounts in all samples. There were not detected ochratoxins A and B, cyclopiazonic acid, penicillic acid, citrinin and patulin in the analyzed fungal extracts. Besides, as biomass yield and nutritional content were successfully modulated by changing fungal culture media composition, the process showed good potential for industrial development. These results show *P. sclerotiorum* as a singular producer of nutritious and safe biomass from which a novel single cell protein food can be developed.

1. Introduction

Single cell protein or microbial protein is a sustainable alternative feed/ food source to supply nutritional needs of a growing world population once it has high protein content while requiring less natural resources and land use than agriculture and livestock (Anupama, 2000; Matassa et al., 2016). Fungi are good producers of a nutritionally complete biomass and useful metabolites such as enzymes when grown in a diversity of substrates as cheap as industrial waste (Tisma et al., 2012). Mycoprotein Quorn, a fungal single cell based product, developed as a meat substitute by Marlow Foods in the 1980's, has been the most noted example of microbial protein in terms of tangible profits and consumer acceptance. This biotechnological product is made from filamentous fungus *Fusarium venenatum* A3/5 (ATCC PTA-2684) mycelium which is grown in continuous flow process in airlift bioreactor and mixed with other ingredients to provide desired texture and flavour (Wiebe, 2004). It is likely that other fungal species exist, notably in Brazil, one of the most biodiverse countries in the world, suitable for mycoprotein production in terms of biomass yield and nutrient contents (Carvalho et al., 2010; Pylro et al., 2014) that have not been fully exploited for this purpose so far. The present study aimed to assess biomass yield and nutritional composition of the fungus *P. sclerotiorum* isolated from Brazilian soil, in order to determine its potential use as a single cell protein food. Additionally, mycotoxins production by *P. sclerotiorum*, which would limit the use of its biomass as a food, was evaluated.

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2. Methods

2.1 Fungal origin and cultivation

Filamentous fungus *P. sclerotiorum* isolated from soil samples collected in Minas Gerais, Brazil and deposited at Biotechnology and Bioassays Laboratory, Department of Chemistry, Universidade Federal de Minas Gerais (UFMG, Brazil) was used in this work. This fungus was grown and allowed to reach sporulation in test tubes containing potato dextrose agar (PDA) at room temperature for 7 days. After that, the spores were suspended in a Tween 80/distilled water solution 0.5%. Neubauer chamber was used to count the number of spores per milliliter of solution. Spores suspension was added in Erlenmeyer Flasks (1 mL per flask) containing liquid culture media with varied glucose, peptone and sodium chloride, as shown in Table 1, and fixed amounts of monopotassium phosphate and magnesium sulfate. Biomass growth occurred at room temperature (~ 28°C) during 23 days, as well as secondary metabolites production. This time is enough to allow mycotoxin production.

2.2 Biomass yield and fermented broth extraction

P. sclerotiorum biomasses were vacuum-filtered and frozen at -18°C until analysis. Biomasses were oven-dried at temperature of 105°C for 8 h and dry weights were recorded, allowing comparison of biomass yields from different treatments. Nutritional analysis was carried out in triplicate samples. Fermented broths were extracted with solvent ethyl acetate through repeatedly vigorous agitation in separatory funnels allowing complete organic/aqueous layers separation. Organic layers were submitted to rotary evaporation to produce extracts which were used for mycotoxin analysis.

2.3 Ash content and minerals quantification

Around 2 g of each biomass sample were placed in porcelain crucibles and incinerated in Bunsen burner until smoke was completely vanished. After that, samples were incinerated in muffle furnace at 560°C for 8 h and weighted. Ash that showed black spots indicating incomplete material carbonization returned to the muffle at 560°C for 6 h after which they were weighted (AOAC, 2012). Ash content was given by Eq(1):

$$AC = \frac{(a - c) \times 100}{w - c} \quad (1)$$

AC = ash content
a = weight of ash + weight of crucible
w = sample weight + weight of crucible
c = weight of crucible

Ash samples obtained in previous step (0.05 g) were solubilized in 5 mL of nitric acid/deionized water solution 60% under heating and transferred to glass flasks. The samples were analyzed by atomic absorption spectroscopy for Ca, Mg, Zn and Fe quantification.

2.4 Protein content

Around 0.25 g of each biomass sample was placed in a digestion tube and 0.3 g of a mixed catalyst containing titanium dioxide, copper sulfate and potassium sulfate (0.3:0.3:6) was added. Sulfuric acid (5 mL) was added to each tube which were placed on a digestion block and temperature was gradually raised until 375°C, being maintained in that temperature for 8 h. After reaching room temperature, the samples were transferred to digestion tubes from Kjeldahl apparatus with 50 mL of deionized water and 3 drops of phenolphthalein solution were added. The digestion tubes were coupled to Kjeldahl apparatus and 30 mL of a sodium hydroxide solution 30% were slowly added to the solution containing the sample. The system was turned on and remained that way until 75 mL of distilled solution was obtained in an Erlenmeyer flask containing 10 mL of sulfuric acid 0.05 M and 3 drops of methyl red setted up after the condenser. The distilled was titrated with sodium hydroxide solution 0.1 M and the volume required to titration recorded (Adapted from AOAC, 2012).

Protein content was given by Eq(2):

$$PC = \frac{v \times 0.14 \times f}{w} \quad (2)$$

PC = protein content

v = sulfuric acid volume added – sodium hydroxide volume consumed in titration
f = conversion factor = 4.38
w = weight of sample

2.5 Fatty acids profile

Around 0.5 g of each sample (dried biomass) was grinded and extracted with a chloroform/methanol (2:1) mixture, according to Folch et al. (1957) methodology. The extracts were hydrolyzed and methylated following methodology suggested by Christie (1989) and analyzed by gas chromatography (CG) SGE BP20 column 15 m x 0.22 mm x 0.25 µm, temperature gradient 100°C, 0 min, 7°C/min until 220°C; injector (split 1/30) 250°C and detector 260°C. Hydrogen was used as mobile phase and volume of injection was 2 µL. The identification of peaks was made by comparison with standard FAME MIX C14-22 (Supelco cat. No.18917).

2.6 Mycotoxins analysis

Extracts were diluted in HPLC grade methanol (1 mg/ mL), vortexed, filtered with 0.22 µm syringe filter and analyzed by Ultra Performance Liquid Chromatography-tandem Mass Spectrometer (UPLC-MS/MS) Acquity I-class C18 – 2.1 x 100 mm x 1.7 µm, column oven 40°C and autosampler 10°C; Mass Spectrometer Xevo TQ-S ESI Triple Quadrupole MRM positive. For transition 404.1>239 it was used cone voltage of 30 V and collision energy of 23 V. For transition 404>221 it was used cone voltage of 30 V and collision energy of 35 V. A standard solution of ochratoxin A (OTA) was injected in the same conditions. The same samples were injected in a Liquid Chromatography/Mass Spectrometer (LC/MS-IT-TOF 225-07100-34) Shimadzu with degasser in line DGU-20A3, two pumps LC-20AD, autosampler SIL-20A, communication module CBM-20A. The heating block temperature was 250°C, capillary voltage was 4.5 kV, CDL temperature 200°C and detector voltage was 1.76 kV. Mass/ charge ratios (m/z) obtained in mass spectra were compared with values of the principal monoisotopes of ochratoxins A and B, ciclopiazonic acid, penicilic acid, citrinin and patulin, the main mycotoxins produced by *Penicillium* fungi.

3. Results and discussion

3.1 Biomass yield and nutritional composition

Biomass yields, ash, and protein content are shown in Table 1. Biomass yields ranged from 1.89 g to 7.80 g. Culture media with higher glucose concentrations (2,4,6 and 8) yielded greater amounts of biomass compared to those with lower glucose concentrations (1,3,5,7,9,10 and 11), highlighting the importance of carbon sources to mycelial development. Ash percentages ranged between 3.55% and 18.11% on a dry matter basis (d.m.). Media 1,2,3 and 4 presented lower ash content probably due to NaCl, once ions sodium and chlorine are ash constituents. Carvalho et al. (2010) reported similar ash percentages in *P. sclerotiorum* (4.64%), *Penicillium janthinellum* (4.76%), *Rhizopus stolonifer* (5.91%) and *Syncephalastrum racemosum* (9.08%) biomasses (d.m.) cultivated under similar conditions. Values found in the present study are similar to ash contents reported by Wang et al. (2014) for edible wild mushrooms from China which ranged from 4.3% in *Lentinula edodes* and 13.3% in *Lactarius volemus,* based on d.m. Protein content ranged from 21.56% to 42.30% on a d.m. basis. Similar percentages were also found by Carvalho et al. (2010) for biomasses of *P. sclerotiroum* (34.3%), *P. janthinellum* (37.47%), *R. stolonifer* (26.81%) and *S. racemosum* (31.10%) and by Wiebe (2002) for mycoportein Quorn (44% d.m.). USDA (2017) also reports similar protein contents for food traditionally consumed as vegetable origin protein like raw mature seed soybeans (39.89% d.m.), tofu (29.74% d.m.), raw peanuts (27.59% d.m.), raw lentil (26.85% d.m.), raw chickpeas (24.18% d.m.) and pistachio nuts (21% d.m.), dairy products as fluid cow and goat milk (33.43% and 27.45% d.m., respectively) and mushrooms as raw white mushroom (40.93% d.m.), raw oyster mushroom (30.59% d.m.) and raw shiitake mushroom (21.83%). These results reveal *P. sclerotiorum* biomass as a potentially valuable nutrient source for human diet, especially regarding its protein content.
Mineral content are shown in Table 2. Macromineral content (Ca and Mg) was quite variable, meanwhile micromineral content did not differ statistically between treatments. Biomass 11 presented the highest Ca content and biomass 1 presented the highest Mg content. Dietary Reference Intakes (DRI) from USDA recommends daily intakes for adults between 1000 - 1300 mg (Ca), 240 - 420 mg (Mg), 8 - 11 mg (Zn) and 8 – 18mg (Fe) (DRI, 2005). Mineral contents found in the present study are similar or slightly lower to mineral contents of some food commonly consumed as a source of such nutrients as cashew nuts (Ca 37 mg/100g and Mg 292 mg/100g), peanuts (Ca 92 mg/100g and Mg 168 mg/100g), pistachio nuts (Zn 2.20 mg/100g and Fe 3.92 mg/100g) and dried milk (Fe 0.47 mg/ 100g and Zn 3.34 mg/100g) (USDA, 2017).

Table 1: Culture media composition (g/ L) and their respective biomass yields (g), ash (%) and protein (%) content on a dry matter basis (d.m.) (means ± standard deviations).

Medium	Composition	Biomass yield	Ash	Protein
1	1 (25 G; 25 P; 0 NaCl)	1.89 ± 0.05 e	5.02 ± 0.07 d	41.88 ± 5.96 a
2	2 (100 G; 25 P; 0 NaCl)	5.46 ± 0.19 bc	3.55 ± 0.51 d	29.44 ± 3.28 bc
3	3 (25 G; 100 P; 0 NaCl)	4.25 ± 0.32 d	6.16 ± 0.27 d	42.30 ± 2.94 a
4	4 (100 G; 100 P; 0 NaCl)	7.80 ± 0.25 a	5.35 ± 0.56 d	36.18 ± 7.08 ab
5	5 (25 G; 25 P; 25 NaCl)	2.12 ± 0.01 e	18.11 ± 1.30 a	33.97 ± 1.14 ab
6	6 (100 G; 25 P; 25 NaCl)	6.19 ± 0.06 b	10.52 ± 0.50 c	21.56 ± 3.05 c
7	7 (25 G; 100 P; 25 NaCl)	4.27 ± 0.18 cd	14.32 ± 0.39 b	34.41 ± 1.00 ab
8	8 (100 G; 100 P; 25 NaCl)	6.54 ± 1.18 b	11.57 ± 1.43 c	32.05 ± 1.04 b
9	9 (62.5 G; 62.5 P; 12.5 NaCl)	4.97 ± 0.29 cd	11.00 ± 1.76 c	36.78 ± 1.90 ab
10	10 (62.5 G; 62.5 P; 12.5 NaCl)	4.52 ± 0.12 cd	9.80 ± 0.83 c	31.83 ± 0.82 b
11	11 (62.5 G; 62.5 P; 12.5 NaCl)	4.46 ± 0.37 cd	9.17 ± 0.46 c	30.89 ± 0.85 bc

Different letters in the same column indicate significant differences ($p<0.05$ Tukey's test).
(G) glucose, (P) peptone, (NaCl) sodium chloride.

Table 2: Ca, Mg, Zn and Fe contents (mg/ 100g biomass) on a dry matter basis (d.m.) (means± standard deviations).

Medium	Ca	Mg	Zn	Fe
1	69.3 ± 7.9 bc	154.2 ± 9.6 a	1.4 ± 0.2 a	1.2 ± 0.3 a
2	22.3 ± 2.8 cd	116.2 ± 13.2 abc	0.9 ± 0.1 a	0.9 ± 0.0 a
3	81.4 ± 4.6 b	125.6 ± 9.5 abc	1.7 ± 0.3 a	1.6 ± 0.1 a
4	52.3 ± 1.0 bcd	103.4 ± 11.1 bc	1.4 ± 0.0 a	1.1 ± 0.3 a
5	71.7 ± 7.2 b	145.4 ± 8.9 ab	2.0 ± 0.9 a	1.3 ± 0.9 a
6	11.9 ± 4.3 d	87.9 ± 13.1 c	1.4 ± 0.4 a	0.6 ± 0.0 a
7	88.8 ± 4.7 b	100.3 ± 8.4 c	3.0 ± 0.2 a	1.6 ± 0.6 a
8	92.6 ± 18.1 ab	87.2 ± 7.7 c	2.1 ± 0.1 a	1.7 ± 0.4 a
9	58.4 ± 27.6 bcd	96.8 ± 13.4 c	2.2 ± 1.3 a	0.8 ± 0.2 a
10	62.0 ± 17.9 bc	104.0 ± 17.6 bc	1.6 ± 0.3 a	1.4 ± 0.1 a
11	137.5 ± 5.9 a	118.2 ± 0.8 abc	2.8 ± 0.7 a	1.1 ± 0.0 a

Different letters in the same column indicate significant differences ($p<0.05$ Tukey's test).

Fatty acids analysis performed in the present study revealed the presence of oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acids, which are unsaturated fatty acids. Linoleic and linolenic acids are polyunsaturated fatty acids (PUFA) also known as omega-6 and omega-3 respectively and their consumption have been associated to a beneficial anti-inflammatory activity to human health. Besides, replacing saturated fatty acids (SFA) by PUFA and monounsaturated fatty acids (MUFA) in human diet has been linked to a decrease of low density lipoprotein (LDL) cholesterol and total cholesterol (TC) to high density lipoprotein (HDL) cholesterol ratio, thus decreasing risk of coronary heart diseases (CHD) (Wang and Hu, 2017).

3.2 Mycotoxins

Mycotoxins were not detected in any sample analysed in this work. Chromatograms of the extracts fortified with OTA standard showed signals in retention time of 8.6 min., as exemplified by Figure 1, thus discarding possible methodological and instrumental fails. Signals appearing in the same retention time in the chromatograms of the samples represent noise instead of OTA presence, as shown in Figure 2.

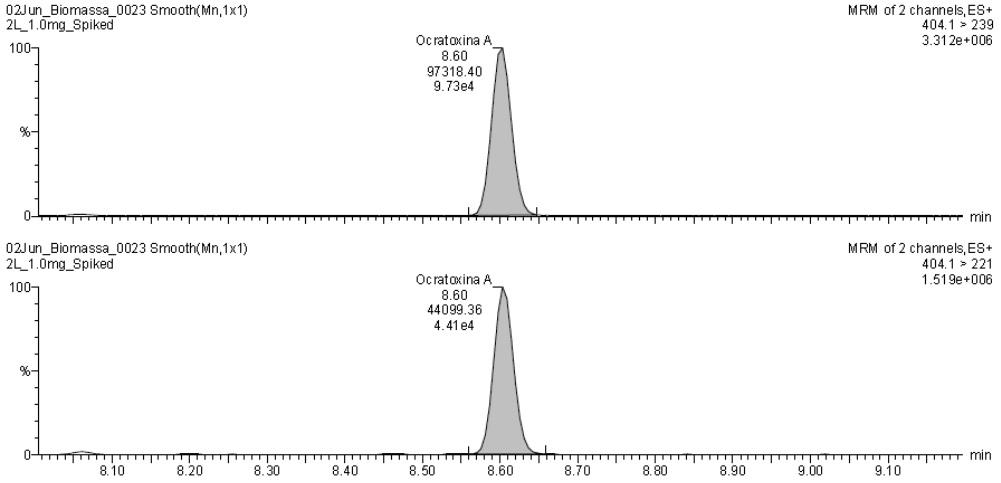

Figure 1: Chromatogram (UPLC) of extract from culture medium 2 fortified with OTA.

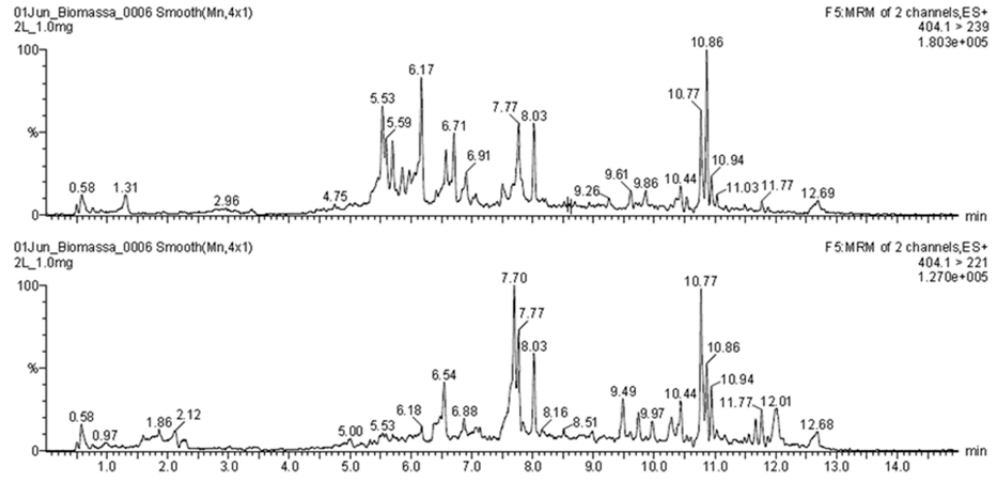

Figure 2: Chromatograms (quantification and confirmation transitions) of the extract from culture medium 2.

Mass spectra obtained in this study are exemplified in Figure 3. Mass-to-charge ratios in mass spectra of all analysed samples were not equivalent to mass-to-charge ratios of the main monoisotopes of ochratoxins A and B, ciclopiazonic acid, penicilic acid, citrinin and patulin molecules, indicating the absence of these molecules in *P. sclerotiorum* extracts. A main requirement for a substance/organism to be used as/in food products is to prove its safety based on scientific procedures (FDA, 2015). Concerning to fungal species utilization as/in food, the absence of mycotoxin production is an important step to evidence its safety.

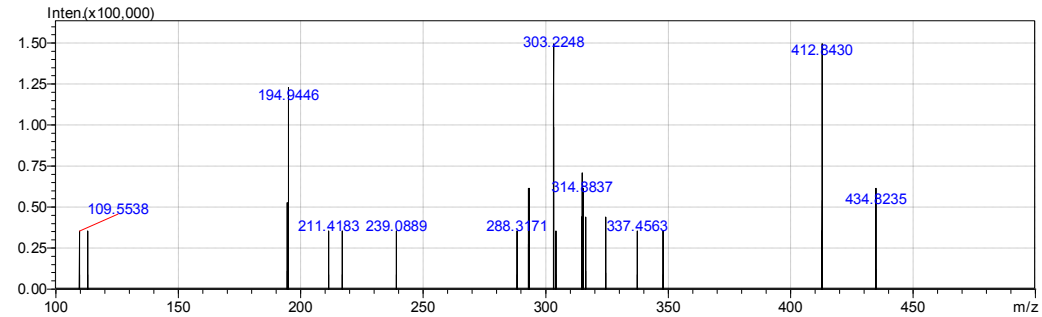

Figure 3: Mass spectrum of the extract from culture medium 2.

4. Conclusions

P. sclerotiorum biomass was proven nutritionally rich and this fungal species has not so far showed mycotoxin production, which indicates the potential of a food product development from its biomass. Future research is required in order to determine the ideal condition for biomass cultivation once it was proved that changes in culture media influences yield and nutrient contents. Also, it is necessary to evaluate other aspects regarding biomass toxicity as well as the costs of industrial scale production and processing.

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