DOI: 10.1111/jfq.12137

Journal of Food Quality

Journal of Food Quality ISSN 1745-4557

BIOMASS AND STEROL PRODUCTION FROM VEGETAL SUBSTRATE FERMENTATION USING *AGARICUS BRASILIENSIS*

JOÃO BENHUR MOKOCHINSKI[1], VANESSA SOVRANI[2], HERTA STUTZ DALLA SANTA[2], MARIA LURDES FELSNER[1], ALEXANDRA CHRISTINE HELENA FRANKLAND SAWAYA[3], PEDRO PABLO GONZÁLEZ-BORRERO[4], GIOVANA ANCESKI BATAGLION[5], MARCOS NOGUEIRA EBERLIN[5] and YOHANDRA REYES TORRES[1,6]

Departamento de [1]Química, [2]Engenharia de Alimentos, [4]Física, Universidade Estadual do Centro-Oeste (UNICENTRO), Guarapuava, Paraná, Brazil, [3]Curso de Farmácia e Programa BTPB, Departamento de Biologia Vegetal, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Campinas, São Paulo, Brazil,
[5]Laboratório ThoMSom de Espectrometria de Massas, Instituto de Química, Universidade Estadual de Campinas (UNICAMP), Campinas, São Paulo, Brazil

[6]Corresponding author.
TEL: +55-42-3629-8325;
FAX: 55-42-3629-8301;
EMAIL: ryohandra@yahoo.com

Received for Publication January 24, 2014
Accepted for Publication January 3, 2015

10.1111/jfq.12137

ABSTRACT

Agaricus brasiliensis was cultivated in vegetal substrates through submerged and solid-state fermentation. We aimed to determine which combination of fermentation methods and substrates would allow greater production of biomass and sterols for applications in nutraceutical foods. Six vegetal substrates were tested: wheat and malt grains, apple, grape and pineapple pomaces, and pineapple peel. Average ergosterol and total sterol levels ranged from 324 to 1,267 μg/g and from 701 to 2,659 μg/g, respectively. The extraction of ergosterol from biomass was optimized by experimental design. As a consequence, a simple and efficient extraction procedure was achieved. Seven sterols were identified by gas chromatography–mass spectrometry in fermented samples, and ergosterol and β-sitosterol were the most abundant. All substrates allowed a good development of *A. brasiliensis* mycelium with outstanding results for malt in submerged phase.

PRACTICAL APPLICATIONS

The vegetal substrates wheat grains, malt grains, apple pomace, grape pomace, pineapple pomace and pineapple peel promote a good development of *Agaricus brasiliensis* mycelium through solid-state fermentation and submerged fermentation techniques. Some of those substrates are discarded by the food industry. The results in this study indicate that vegetal residues from food industry could be effectively used as substrates to produce edible and medicinal mushrooms and sterols. Production of mycelium from the methods employed proved to be simple, fast, reproducible and efficient. Biomass production using residues from food industry adds value to these residues and at the same time solves disposal problems of agricultural by-products.

INTRODUCTION

Agaricus brasiliensis is a medicinal and edible mushroom, traditionally used in Brazil, Japan and other countries for prevention of diseases such as diabetes, cancer, hyperlipidemia, arteriosclerosis and chronic hepatitis (Zou 2006). Its immunostimulant properties and strong antitumor activity (Stamets 2000; Wasser 2011) have made the production of this mushroom of interest for pharmaceutical and food industries, which seek to obtain the bioactive compounds from this mushroom or to develop nutraceutical products (Okwulehie *et al.* 2007). Polysaccharides and ergosterol are among the most studied bioactive molecules produced by *A. brasiliensis* (Hamedi *et al.* 2007; Leifa *et al.* 2007; Lima

et al. 2008). Takaku *et al.* (2001) showed the relationship between ergosterol from *A. brasiliensis* and their antitumor activity by the inhibition of angiogenesis and reduction in tumor growth. Furthermore, ergosterol is a well-known chemical marker for fungal mass because it is one of the most abundant sterols found in mushrooms of different species (Czub and Baginski 2006). In the human body, it plays an important role as a precursor of vitamin D_2, which is formed in response to ultraviolet radiation (sunlight) on sterols found in the skin (Mattila *et al.* 2002; Jasinghe and Perera 2005).

There is an increasing worldwide demand for ergosterol in order to produce pharmaceutical-grade, food-grade and beverage-grade vitamin D_2. Because of this demand, mycelium fermentation was recently assessed for optimizing ergosterol and/or polysaccharide production by *A. brasiliensis* (Fan *et al.* 2003; Zou 2005; Gao and Gu 2007; Hamedi *et al.* 2007). These important substances present in the fruiting body are also found in smaller amounts in the mycelium and spores. As the production of the fruiting body takes several months and needs larger spaces (Smith *et al.* 2002; Hamedi *et al.* 2007), fermentation techniques such as solid-state fermentation (SSF) and submerged fermentation (SF) have been developed to improve mycelium production. SF is reported as the best process to obtain fungal mycelium and its biomolecules because of the shorter fermentation time (7 days), less space requirement and better control of fermentation parameters (Fan *et al.* 2003; Gao and Gu 2007; Leifa *et al.* 2007). Alternatively, SSF provides a good dwelling environment for fungi, requires low capital investment and reduces product contamination due to low amount of water (Pandey 2008). Therefore, SSF is the technique of choice for large-scale production (Dalla Santa *et al.* 2012).

Vegetal residues from the food industry have been included in food as a source of fibers. Recently, fermented residues of mycelium of edible medicinal mushrooms have been used in formulations of new nutraceutical foods (Dalla Santa *et al.* 2012). The substrate and the type of fermentation interfere in the metabolic pathway of mushrooms and, consequently, in mycelium and bioactive molecules production. Therefore, this study aimed to determine which combination of vegetal residue and type of fermentation (SSF or SF) would provide the best development of *A. brasiliensis* mycelium and the highest level of sterols. All the fermented substrates were extracted and their ergosterol content was determined by high-performance liquid chromatography (HPLC). Furthermore, the amount of total sterols was quantified by UV-vis technique, and the chemical profile of the extracts was compared by gas chromatography–mass spectrometry (GC-MS).

MATERIAL AND METHODS

Microorganism and Inoculum Preparation

The strain of *A. brasiliensis* (LBC10/UNICENTRO) was previously isolated from a commercial culture and was kept in potato dextrose agar slants and subcultured every 3 months. The standard culture medium for inoculum preparation was composed of glucose (20 g/L), yeast extract (3.95 g/L), $MgSO_4 \cdot 7H_2O$ (0.30 g/L), and $K_2HPO_4 \cdot 3H_2O$ (0.50 g/L); pH was adjusted to 6.0 (±0.2) (Fan *et al.* 2003). For inoculation procedure, five pieces (1 cm^2) of mycelia were transferred to 50 mL of seed culture medium and incubated at 30C on a rotary shaker (120 rpm) for 7 days. The inoculum was prepared as described by Dalla Santa *et al.* (2010). The mycelium was filtered (mesh of 0.5 mm^2), washed with 50 mL of distilled sterilized water and inoculated in 500 mL of the standard medium and incubated. After filtration (mesh 0.5 mm^2) and washing with 500 mL of distilled sterilized water, this suspension of mycelium was used as inoculum.

Substrates and Cultivation

Fermentations were performed in triplicate for each of the six different substrates: wheat grains, malt grains, apple pomace, grape pomace, pineapple pomace and pineapple peel. *A. brasiliensis* was grown in solid (SSF) and submerged phase (SF). For SF, all substrates were dried and ground to a fine powder (<60 mesh) in a manual mill where 5% of substrates were employed in fermentations. The pH was set to 6.0 (±0.2) with 0.1 N NaOH and the medium was sterilized at 121C for 15 min.

The SSF was conducted with a substrate particle size between 1.00 and 1.50 mm and pH was adjusted to 6.0 (±0.2) with $CaCO_3$ solution (10% w/v). Water was added to obtain the optimum initial humidity for each substrate. The medium was sterilized at 121C for 15 min.

The inoculum was made at 5% (v/v) for SF, and *A. brasiliensis* growth was carried out at 30C with stirring at 120 rpm for 7 days. For SSF, 5% (v/w) was used for the inoculum rate and the incubation for the fungi mycelium growth was carried out for 20 days at 30C in an incubator.

After the incubation time, the SF was filtered and the substrate grown with the mycelia as well as the SSF material were dried at 60C.

Experimental Design for Ergosterol Extraction

An optimization of ergosterol extraction conditions was carried out by experimental design using the software Minitab for Windows version 16.2.2. (Minitab Incorporation, College State,

TABLE 1. FACTORS AND THEIR LEVELS FOR A 2^3 FACTORIAL DESIGN TO OPTIMIZE ERGOSTEROL EXTRACTION FROM FERMENTED *AGARICUS BRASILIENSIS*

Factor level	Heating source	Reagents	Drying agent
–1	Microwave	With 1.0 N HCl and 1.0 M $KHCO_3$	With Na_2SO_4
1	Bath	Without 1.0 N HCl and 1.0 M $KHCO_3$	Without Na_2SO_4

Pennsylvania, USA). A 2^3 factorial design was applied to study the simultaneous effect of the heating source, the use of acid and base, and a drying agent on the extraction yield (qualitative variables). Consequently, eight extraction assays were randomly performed in duplicate to determine which procedure was the most efficient to extract ergosterol from the fermentation medium. In Table 1, experimental parameters and their levels are specified.

The effects for factors considered in the factorial design were calculated by

$$E_f = (\bar{R}_+) - (\bar{R}_-)$$

where $(\bar{R}_+)$and $(\bar{R}_-)$ are differences between mean values for (+) and (–) levels, respectively, for each factor. All factor effects in the amount of ergosterol extracted from *A. brasiliensis* had their statistic significant evaluated at 95% confidence level using the standard error.

Biomass samples were dried and ground into fine powder (<60 Mesh) and initial humidity was determined to report ergosterol per dry mass of *A. brasiliensis*. About 0.2500 g of biomass was weighed for each extraction procedure.

Ergosterol esters were saponified with 2 mL of methanol and 0.5 mL of 2 N NaOH using a microwave or a water bath heating. When the microwave was employed, each test tube screw-thread was tightly capped and sealed with Teflon (Young 1995). The tubes were placed in a plastic bottle for microwaves and heated for 20 s. After cooling at room temperature for 15 min, the tubes were heated again for 20 more seconds (Montgomery *et al.* 2000). Water bath heating was carried out thermostatically at 60C for 1 h.

After the heating periods, samples were cooled at room temperature. The procedure was followed by adding an acid, a base or nothing and subsequently by adding or not a drying agent. Afterward, samples were extracted three times with 2 mL of hexane. The solvent was evaporated at 45C under a flow of air to obtain a dry extract. All extracts were stored at −18C until analysis. The results of the experimental design were evaluated by determining the amount of ergosterol by HPLC.

High-Performance Liquid Chromatography–Photodiode Array Detection (HPLC-PDA) and UV-Vis Quantitation

Ergosterol quantification was carried out by HPLC, as previously described with some modifications (Carvalho *et al.* 2006). A Waters (Waters Technologies do Brasil Ltda, Barueri, SP, Brazil) Series 600 liquid chromatograph equipped with a photodiode array detection system (UV/PDA, Waters 2696) was used. Each extract was dissolved in 2 mL of ethanol. All samples were filtered through a 0.2-μm cellulose acetate membrane before analyzing. A Waters μBondapak C_{18} guard column (20 mm × 3.9 mm × 10 μm) and Macherey-Nagel Nucleosil C_{18} analytical column (250 mm × 4.6 mm × 5 μm) were employed at room temperature. Chromatography was carried out in an isocratic reverse-phase elution mode with 100% ethanol as mobile phase and a flow rate of 0.65 mL/min. A 20 μL sample loop was used. Initially, the PDA detector was programmed to scan from 210 to 400 nm to determine the maximum wavelength for ergosterol analysis, and consequently, 282 nm was chosen for best sensitivity in ergosterol quantification. Ergosterol peaks in extracts were identified based on the comparison of retention time of a pure ergosterol standard and its UV spectra data. Ergosterol was quantified by external standard calibration by comparison of ergosterol peak areas in the samples with that of pure standards at concentrations ranging from 1 to 80 μg/mL.

UV-vis analysis was performed in triplicate on a Varian Cary 50 spectrophotometer. A quartz cuvette with 1 cm of optical path was employed and ethanol was used as a blank. The analytical curve was built by measuring the maximum absorbance at 282 nm of standard ethanolic solutions of ergosterol (5–50 μg/mL). All analytical standards were prepared by dilution from a stock solution at 500 μg/mL.

GC-MS Analysis

Identification of sterols was performed using a gas chromatograph (Agilent 7890A, Agilent Technologies Brasil Ltda, Barueri, SP, Brazil) coupled with a mass spectrometer (Agilent 5975C). Samples were separated on an Agilent capillary column HP-1 (30 m × 0.25 mm × 0.25 μm film thickness). The oven temperature was initially held at 50C for 5 min, and then the temperature was raised to 300C at a rate of 5C/min, followed by isothermal period of 2 min at 300C. Total run time was 57 min. The temperatures of injection port and detector were set at 250 and 300C, respectively. Splitless mode was used with helium as carrier gas at a flow rate of 1.5 mL/min and an injection volume of 1 μL. Sterol constituents were identified based on the mass spectral data and on retention times, using the NIST 2008 library and pure standards. All samples were derived by reaction with 50 μL of BSTFA at 60C for 1 h. The

semi-quantification of the identified compounds was carried out by internal normalization with the area of each compound and the sum of all peak areas corresponding to 100% of area.

Statistical Analysis

Two-way analysis of variance (ANOVA) and 95% confidence interval were applied in order to determine significant differences among ergosterol and total sterol contents produced by *A. brasiliensis* in the different fermentation conditions (different substrates and fermentation types) investigated. Statistical analysis of data was carried out using the statistical software Minitab for Windows v. 16 at 95% confidence level.

RESULTS AND DISCUSSION

Optimization of Extraction Procedure

The results for the factorial design are shown in Table 2 together with the calculated effects. The *t*-test and *F*-test revealed significant effects for all the qualitative variables at 95% significance level ($P < 0.05$). The change between levels for the source of heat indicated that microwave heating promoted a slight improvement in extraction yield and was chosen also due to its simplicity and shorter extraction time. The effect of adding acid/base and a drying agent as well as the interaction between them were extremely significant and had a negative influence on the yield of extraction. The best result (366.79 ± 16.59 μg/g) for ergosterol extraction was achieved in the absence of these factors and with microwave-assisted extraction as a result of lesser sample handling steps combined with the higher extraction efficiency of microwaves. Finally, the extraction procedure does not need more than the saponification and liquid–liquid partition steps. Therefore, all ergosterol extractions were performed in the way depicted in Fig. 1.

Different methods have been reported to extract ergosterol from mushrooms; however, there is still no consensus on which method is the best to be employed (Seitz *et al.* 1977; Young 1995; Mattila *et al.* 2002; Tardieu *et al.* 2007; Yuan *et al.* 2007). Some methods have described the use of large amounts of samples (in grams), several steps of refluxing, saponification or sonication and drying agents (Ruzicka *et al.* 1995). Alternatively, Young (1995) proposed a microwave-assisted method, which uses small amounts of samples, small reagent volumes and is faster than the conventional routine methods. Another recently explored technique is pressurized liquid extraction, which offers the advantage of allowing automated sample handling and reducing solvent consumption. However, it is an expensive technique and is not available in all laboratories (Li *et al.* 2004).

TABLE 2. RESULTS FOR THE FACTORIAL DESIGN 2^3 TO OPTIMIZE ERGOSTEROL EXTRACTION FROM FERMENTED *AGARICUS BRASILIENSIS* AND MEAN CALCULATED EFFECTS

Heating source	Reagents	Drying agent	Ergosterol*§ (Mean ± SD) (μg/g)
–1	–1	–1	126.72 ± 4.67[a]
1	–1	–1	121.03 ± 1.84[a]
–1	1	–1	205.86 ± 4.84[b]
1	1	–1	191.08 ± 5.57[b]
–1	–1	1	210.04 ± 3.27[b]
1	–1	1	199.41 ± 2.72[b]
–1	1	1	366.79 ± 16.59[c]
1	1	1	334.14 ± 16.67[d]

Effects	Estimate‡ ± Standard error†
Average	219.38 ± 0.66
Main effects	
Heating source (1)	15.94 ± 1.32
Acid and base (2)	–110.16 ± 1.32
Drying agent (3)	–116.42 ± 1.32
Two-factor interactions	
(1) × (2)	7.78 ± 1.32
(1) × (3)	5.71 ± 1.32
(2) × (3)	–35.78 ± 1.32
Three-factor interactions	
(1) × (2) × (3)	3.23 ± 1.32

* Data are represented as mean values ± standard deviation of two independent quantifications and expressed per gram of substrate.
† Standard errors were calculated from standard deviation.
‡ Confidence intervals at the 95% level were calculated multiplying the standard-errors by $t_{(8,0.025)} = 2.306$ and effects with estimates higher than 3.044 are significant at 95% confidence level.
§ Experiments with equal letters do not present significant differences between ergosterol mean values.

Several procedures in the extraction of ergosterol from biomass have been reported in the scientific literature, and in this study, the extraction of ergosterol from *A. brasiliensis* was optimized by experimental design. As a consequence, an efficient and few-step procedure for ergosterol extraction was achieved.

Determination of Ergosterol Content Produced by *A. Brasiliensis* Grown in Different Vegetal Substrates Through SSF or SF

HPLC-PDA and UV-Vis Method Validation. Analytical external calibration curves were built by injecting each standard solution in triplicate. To determine the regression equations and check the linearity in the concentration range investigated, a linear regression analysis at 95% confidence level was carried out. Additionally, to verify if the linear model was suitable to explain the relationship between area

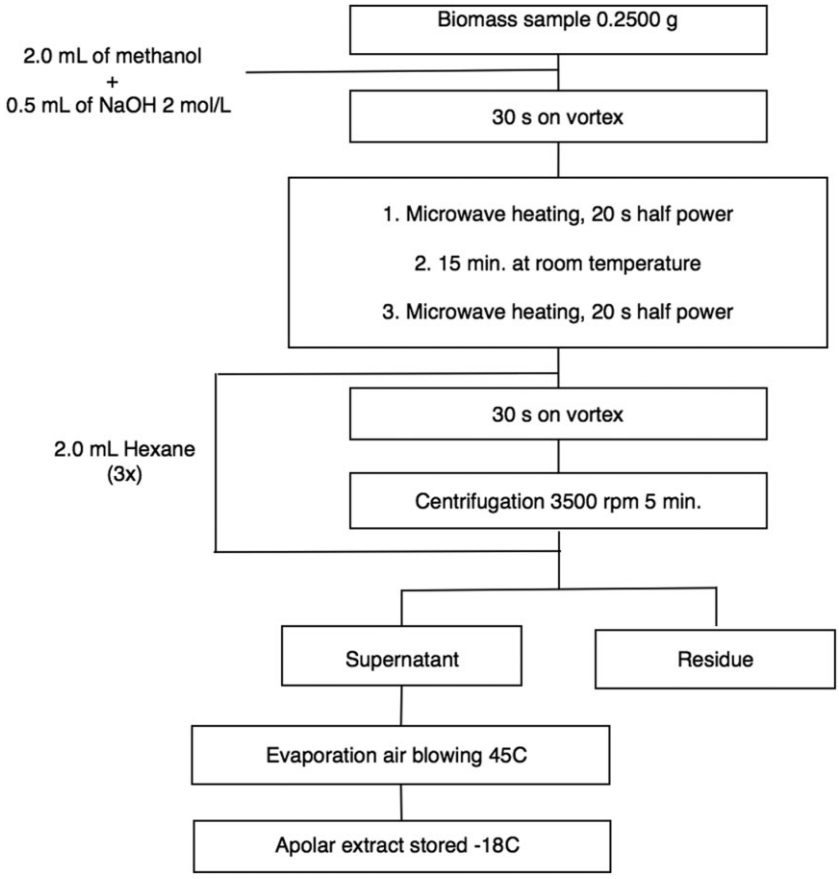

FIG. 1. OPTIMIZED STEPS FOR ERGOSTEROL EXTRACTION FROM DIFFERENT UNFERMENTED AND FERMENTED SUBSTRATES

or absorbance and concentration, the significance of the linear regression and the residual graphs obtained was analyzed. The regression equation for HPLC ergosterol determination was Area = 40472 + 41203C ($R^2 = 99.2\%$) and for UV-vis determination was Absorbance = –0.0265 + 0.0219C ($R^2 = 99.4\%$). These regression equations were considered linear because the determination coefficient (R^2) ranged from 99.2 to 99.4%, the linear regression was significant ($P < 0.05$) and did not identify any lack of fit of linear model ($P > 0.05$). Furthermore, the analysis of residual graphs of the linear regression model (data not shown) showed a random pattern, indicating that the linear model was well adjusted to the experimental data. Selectivity was granted by comparing retention time (7.35 min) and UV spectrum from ergosterol chromatographic peak in the standard and in extracts. By standard addition tests, the selectivity of the analytical method was confirmed since the other components of the extracts did not co-elute with ergosterol and did not interfere in its determination. The limits of detection (LOD) and limits of quantification (LOQ) were calculated from parameters of the analytical curves. LOD was calculated based on $3 \times SD/m$, where m is the slope of the calibration curves and SD is the standard deviation of the linear coefficient. The same way LOQ was calculated by $10 \times SD/m$. LOD and LOQ were 1.53 and 5.09 μg/mL, respectively, for HPLC-PDA and 1.28 and 4.28 μg/mL, respectively, for UV-vis.

Accuracy and intraday precision were appraised by recovery experiments. Biomass was spiked with 1 mL of ergosterol standard solutions at concentrations of 5.0, 25.0 and 50.0 μg/mL. All spiked samples were extracted and quantified by HPLC-PDA and UV-vis, and the recoveries for ergosterol were between 97.61 and 99.88% for HPLC and between 94.89 and 105.26% for UV-vis (Table 3).

The results from recovery experiments (Table 3) showed high recovery percentages for ergosterol determinations by

TABLE 3. RECOVERY EXPERIMENTS FOR ERGOSTEROL DETERMINATION BY HPLC-PDA AND UV-VIS

	HPLC		UV-vis	
Ergosterol spiked (μg)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
5.00	99.88	25.63	94.89	24.24
25.00	99.85	16.42	102.74	16.24
50.00	97.61	15.30	105.26	14.81

HPLC, high-performance liquid chromatography; PDA, photodiode array; RSD, relative standard deviation.

both HPLC-PDA and UV-vis, which also demonstrated the effectiveness of the simplified extraction procedure optimized in this study. The analytical quantitative method was accurate and sensitive to small variations in ergosterol concentration. According to Brito *et al.* (2003), it is possible to relate the recovery value as a function of the level of the analyte in samples; if this value is ≥0.1%, the accepted recovery range must be between 95 and 105%. Based on that, our results were within the acceptable range. However, a relative high uncertainly of 25% was found for intraday precision at the lowest concentration level. The high relative standard deviation (RSD) values could be due to the complexity of the matrix under investigation, fermentation media from diverse vegetal substrates.

Comparison of the Ergosterol Levels Produced by *A. Brasiliensis* Grown in Different Vegetal Substrates and Different Fermentation Techniques. The HPLC-PDA quantification of ergosterol resulted in chromatograms with a narrow ergosterol peak, good resolution and short retention time (7.35 min) (see Supporting Information Fig. S1). The chromatographic behavior indicated that all fermentations were reproducible in both SF and SSF. The results for ergosterol content are graphically represented in Fig. 2 and have the background value of ergosterol discounted for malt and pineapple peel (substrates with a low amount of ergosterol before the fermentation process). For all other substrates, the initial amount of ergosterol was under the LOD, confirming that ergosterol can be a useful tool to estimate the fungi growth (Seitz *et al.* 1977; Newell and Fallon 1991; Montgomery *et al.* 2000; Tardieu *et al.* 2007).

HPLC-PDA quantification of ergosterol was compared with the spectrophotometric UV-vis determination. Ergosterol levels determined by UV method are greater than those found by HPLC-PDA since absorption at λ282 nm cannot be attributed specifically to ergosterol. Consequently, the levels found by UV-vis determination should be considered as a total amount of sterols in the extracts, a parameter that is also a good quantitative measure of biomass growth of fungi (Mattila *et al.* 2002; Teichmann *et al.* 2007). ANOVA results suggest that the amount of ergosterol and total sterol content were strongly influenced by the type of fermentation ($P < 0.05$) and the type of vegetal substrate ($P < 0.05$) (Table 4). Furthermore, there was a significant interaction between those factors ($P < 0.05$). The results of ergosterol determination by HPLC-PDA showed that average ergosterol contents from malt and grape were higher than those obtained on other substrates considering types of fermentations altogether (Table 4). The average ergosterol content from fermented apple, pineapple peel, pineapple pomace and wheat showed no significant statistical differences at 95% confidence level.

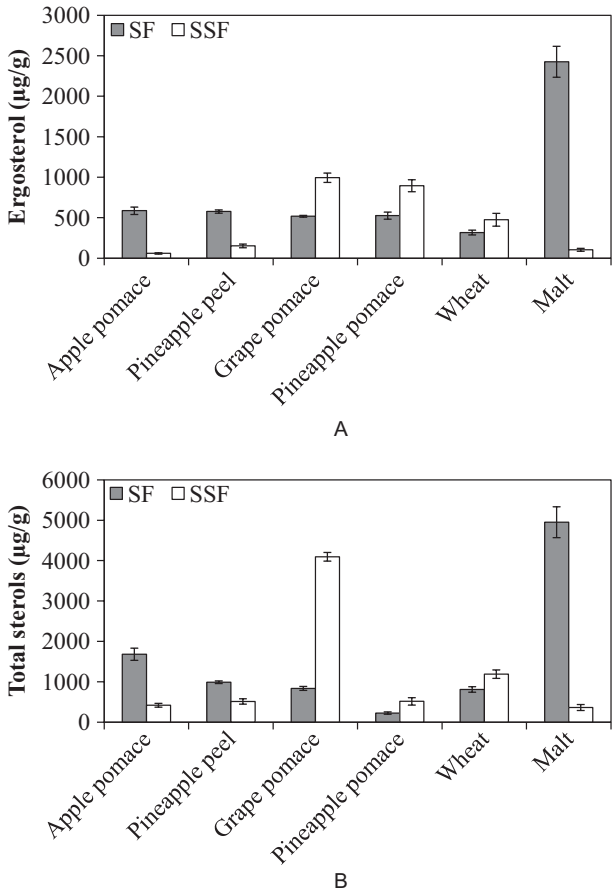

FIG. 2. (A) ERGOSTEROL CONTENT DETERMINED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–PHOTODIODE ARRAY DETECTION AND (B) TOTAL STEROL CONTENT DETERMINED BY UV-VIS IN SEVERAL VEGETAL SUBSTRATES AFTER SUBMERGED (SF) AND SOLID-STATE FERMENTATION (SSF)

Additionally, average ergosterol values obtained from SF were almost twofold higher than the obtained through SSF for apple pomace, pineapple peel and malt (Fig. 2 and Table 4). Remarkably, malt substrate fermented in submerse phase proved to be the best medium to develop the mycelium of *A. brasiliensis*, possibly due to the malting process that exposes nutrients, turning them more accessible to the microorganism. Pineapple peels used in this study were from the juice industry and were formed by peels and a small portion of pulps. These pulps have fermentable sugars that dissolve more readily in the liquid medium and stimulate the development of the mycelium at SF. Conversely, the SSF of grape pomace (and to a lesser extent pineapple pomace) produced higher ergosterol and total sterol contents than the SF. Grape pomace residues had been previously fermented in wine production and therefore have low content of fermentable sugars if compared, e.g., with apple residues. In addition, the high fiber content of pomaces

TABLE 4. RESULTS OF THE TWO-WAY ANALYSIS OF VARIANCE FOR ERGOSTEROL CONTENT DETERMINED BY PERFORMANCE LIQUID CHROMATOGRAPHY–PHOTODIODE ARRAY DETECTION AND TOTAL STEROL CONTENT DETERMINED BY UV-VIS*

Factor investigated	Ergosterol content† (μg/g) (95% confidence interval)	Total sterol content† (μg/g) (95% confidence interval)
Fermentation		
SF	862.2 ± 99.7 (835.1–889.3)[a]	1695.7 ± 205.0 (1639.9–1715.5)[a]
SSF	336.4 ± 44.8 (317.3–341.7)[b]	333.9 ± 44.5 (1134.7–1234.3)[b]
Substrate		
Apple pomace	323.7 ± 64.4 (293.3–354.1)[a]	1053.0 ± 155.0 (975.9–1130.1)[a]
Pineapple pomace	365.2 ± 51.7 (339.6–391.0)[a]	752.7 ± 58.9 (723.4–782.0)[b]
Grape pomace	757.4 ± 58.5 (728.3–786.5)[b]	2468.0 ± 396.0 (2271.1–2664.9)[c]
Pineapple peel	377.6 ± 37.5 (396.2–359.0)[a]	701.1 ± 50.0 (682.2–732.0)[b]
Wheat	397.3 ± 23.9 (385.4–409.2)[a]	1001.5 ± 50.3 (976.5–1026.5)[a]
Malt	1267.0 ± 283.0 (1126.3–1407.7)[c]	2659.0 ± 560.0(2380.5–2937.8)[c]

* Different letters indicate significant statistical differences at a 95% confidence level.
† Data are represented as mean values ± standard error.

(grape and pineapple pomaces) provides good aeration and access to nutrients because the particle size is adequate and facilitates the development of mycelium in SSF (Cristi 1999).

By inspection of Fig. 2, it is also possible to verify that the total sterol content obtained by UV-vis showed the same trend observed for the content of ergosterol with regard to the type of vegetal substrate, fermentation and interaction between those variables. However, the differences between the mean levels of total sterols for the type of fermentation, SF and SSF, or among the types of substrates were less pronounced (Table 4).

Application of GC-MS on Sterol Identification and Semi-Quantitation

Seven sterols were identified in fermented samples (Table 5). The trend observed for the amount of ergosterol by HPLC quantification was comparable to that found by GC-MS, where SF produced a higher content of ergosterol than SSF. In Table 5, the sterols identified in fermented substrates and their relative abundance are listed.

The most intense peak in the chromatograms of the extract analyses by HPLC-PDA was due to ergosterol because the selected wavelength (282 nm) was optimized for this sterol during method development. Nevertheless, GC-MS analysis showed that β-sitosterol was rather abundant in the majority of the extracts. β-Sitosterol was not detected by HPLC-PDA because its wavelength of maximum absorbance is lower (208 nm) than the wavelength selected for ergosterol quantification (Dutta 2003).

Mattila *et al.* (2002) also determined the sterol composition in some wild and cultivated mushrooms by GC-MS. Ergosterol was quantified in different parts of the mushrooms (pileus, stipe and gill). The results indicated that for most studied species, gills were the richest ergosterol source.

TABLE 5. RELATIVE ABUNDANCES (%) OF STEROLS IDENTIFIED BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY IN SUBSTRATES FERMENTED BY *AGARICUS BRASILIENSIS*

	Relative abundance (%)						
Fermented samples	Dehydroergosterol *m/z* 466	Ergosterol *m/z* 468	Campesterol *m/z* 472	Stigmasterol *m/z* 484	Fungisterol *m/z* 472	β-Sitosterol *m/z* 486	Stigmastanol *m/z* 488
Apple pomace SF	2.18	20.91	4.37	0	2.34	70.21	0
Apple pomace SSF	2.47	5.66	4.78	0	1.59	83.73	0
Pineapple peel SF	1.67	41.96	8.53	5.32	4.47	30.89	7.17
Pineapple peel SSF	3.99	32.70	11.95	3.94	4.11	34.87	8.43
Grape pomace SF	0.86	16.34	5.00	4.28	3.36	68.23	1.94
Grape pomace SSF	3.23	34.26	5.76	3.33	2.85	50.57	0
Wheat SF	2.68	64.87	9.14	0	5.76	17.55	0
Wheat SSF	3.51	44.11	11.27	2.03	1.04	33.07	4.96
Pineapple pomace SF	1.36	35.67	11.45	0	7.02	37.84	6.66
Pineapple pomace SSF	0,49	12.78	14.76	1.42	1.14	57.27	12.14
Malt SF	2.66	62.39	11.41	0	9.53	14.02	0
Malt SSF	1.77	23.47	20.53	3.92	6.51	43.81	0

SF, submerged fermentation; SSF, solid-state fermentation.

The chemical composition of wild and cultivated mushrooms had a similar profile for all species studied, varying the content of each compound.

CONCLUSIONS

The amount of ergosterol produced by *A. brasiliensis* grown on different substrates showed that all substrates allow the growth of this fungus and are potential substrates for the fermentation process, adding value to residues from food industry. SF and SSF proved to be an efficient way to produce the biomass. The combination of malt substrate and submerged fermentation was the best arrangement for the production of biomass and sterols. Ergosterol and β-sitosterol were the most abundant sterols found on fermented samples.

ACKNOWLEDGMENTS

The authors thank the financial support provided by Financiadora de Estudos e Projetos (FINEP) , Secretaria da Ciência, Tecnologia e Ensino Superior (SETI), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Fundação Araucária (10908/PPP/2006), and also Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the scholarships.

REFERENCES

BRITO, N.M., DE AMARANTE Jr., O.P., POLESE, L. and RIBEIRO, M.L. 2003. Validação de métodos analíticos: Estratégia e discussão. Pesticidas: R.Ecotoxicol. e Meio Ambiente *13*, 129–146.

CARVALHO, J.C., PANDEY, A., OISHI, B.O., BRAND, D., RODRIGUEZ-LEÓN, J.A. and SOCCOL, C.R. 2006. Relation between growth, respirometric analysis and biopigments production from *Monascus* by solid-state fermentation. Biochem. Eng. J. *29*, 262–269.

CRISTI, Y. 1999. Fermentation (industrial): Basic considerations. In *Encyclopedia of Food Microbiology* (R. Robinson, C. Batt and P. Patel, eds.) pp. 663–674, Academic Press, London, U.K.

CZUB, J. and BAGINSKI, M. 2006. Comparative molecular dynamics study of lipid membranes containing cholesterol and ergosterol. Biophys. J. *90*, 2368–2382.

DALLA SANTA, H.S., RUBEL, R., FERNANDES, L.C., BONATTO, J.R., BELLO, S.R., MONTEIRO, M.C., KHALIL, N.M., DALLA SANTA, O.R., SOCCOL, C.R., GERN, J.C. *et al.* 2010. *Agaricus brasiliensis*-enriched functional product promotes in mice increase in HDL levels and immunomodulate to Th1 CD4+T subsets. *A. brasiliensis* functional product and biological benefits. Curr. Trends Biotechnol. Pharm. *4*, 957–970.

DALLA SANTA, H.S., RUBEL, R., VÍTOLA, F.M.D., RODRIGUEZ-LEON, J.A., DALLA SANTA, O.R., BRAND, D., ALVARÉZ, D.C., MACEDO, E.E.F., CARVALHO, J.C. and SOCCOL, C.R. 2012. Growth parameters of *Agaricus brasiliensis* mycelium on wheat grains in solid-state fermentation. Biotechnology *11*, 144–153.

DUTTA, P.C. 2003. *Phytosterol as Functional Food Components and Nutraceutical*, 1st Ed., Marcel Dekker, New York, NY.

FAN, L., SOCCOL, A.T., PANDEY, A., GERMANO, S., RAU, R., PEDROSO, A.A.L. and SOCCOL, C.R. 2003. Production of polysaccharide by culinary-medicinal mushroom *Agaricus brasiliensis* S. Wasser et al. LPB 03 (Agaricomycetideae) in submerged fermentation and its antitumor effect. Int. J. Med. Mushrooms *5*, 17–23.

GAO, H. and GU, W.Y. 2007. Optimization of polysaccharide and ergosterol production from *Agaricus brasiliensis* by fermentation process. Biochem. Eng. J. *33*, 202–210.

HAMEDI, A., VAHID, H. and GHANATI, F. 2007. Optimization of the medium composition for production of mycelial biomass and exo-polysaccharide by *Agaricus blazei* Murill DPPh 131 using response-surface methodology. Biotechnology *6*, 456–464.

JASINGHE, V.J. and PERERA, C.O. 2005. Distribution of ergosterol in different tissues of mushrooms and its effect on the conversion of ergosterol to vitamin D2 by UV irradiation. Food Chem. *92*, 541–546.

LEIFA, F., SOCCOL, A.T., PANDEY, A. and SOCCOL, C.R. 2007. Effect of nutritional and environmental conditions on the production of exo-polysaccharide of *Agaricus brasiliensis* by submerged fermentation and its antitumor activity. Food Sci. Technol -LEB *40*, 30–35.

LI, S.P., LI, P., LAI, C.M., GONG, Y.X., KAN, K.K.W., DONG, T.T.X., TSIM, K.W.K. and WANG, Y.T. 2004. Simultaneous determination of ergosterol, nucleosides and their bases from natural and cultured *Cordyceps* by pressurized liquid extraction and high-performance liquid chromatography. J. Chromatogr., A *1036*, 239–243.

LIMA, L.F.O., HABU, S., GERN, J.C., NASCIMENTO, B.M., PARADA, J.L., NOSEDA, M.D., GONÇALVES, A.G., NISHA, V.R., PANDEY, A., SOCCOL, V.T. *et al.* 2008. Production and characterization of the exopolysaccharides produced by *Agaricus brasiliensis* in submerged fermentation. Appl. Biochem. Biotechnol. *151*, 283–294.

MATTILA, P.H., LAMPI, A.M., RONKAINEN, R., TOIVO, J. and PIIRONEN, V. 2002. Sterol and vitamin D2 contents in some wild and cultivated mushrooms. Food Chem. *76*, 293–298.

MONTGOMERY, H.J., MONREAL, C.M., YOUNG, J.C. and SEIFERT, K.A. 2000. Determination of soil fungal biomass from soil ergosterol analyses. Soil Biol. Biochem. *32*, 1207–1217.

NEWELL, S.Y. and FALLON, R.D. 1991. Toward a method for ensuring fungal instantaneous growth rates in field samples. Ecology *72*, 1547–1559.

OKWULEHIE, I.C., NWOSU, C.P. and JOHNPAUL, O.C. 2007. Pharmaceutical and nutritional prospects of two wild macro-fungi found in Nigeria. Biotechnology *6*, 567–572.

PANDEY, A. 2008. Solid-state fermentation. SciTopics. https://sites.google.com/a/sgbau.ac.in/bioprocess-technology/home/solid-state-fermentation (accessed September 5, 2013).

RUZICKA, S., NORMAN, M.D.P. and HARRIS, J.A. 1995. Rapid ultrasonification method to determine ergosterol concentration in soil. Soil Biol. Biochem. *27*, 1215–1217.

SEITZ, L.M., MOHR, H.E., BURROUGHS, R. and SAUER, D.B. 1977. Ergosterol as an indicator of fungal invasion in grains. Cereal Chem. *54*, 1207–1217.

SMITH, J.E., ROWAN, N.J. and SULLIVAN, R. 2002. Medicinal mushrooms: A rapidly developing area of biotechnology for cancer therapy and other bioactivities. Biotechnol. Lett. *24*, 1839–1845.

STAMETS, P. 2000. Techniques for the cultivation of the medicinal mushroom Royal Sun *Agaricus-blazei Murr*. (Agaricomycetideae). Int. J. Med. Mushrooms *2*, 151–160.

TAKAKU, T., KIMURA, Y. and OKUDA, H. 2001. Isolation of an antitumor compound from *Agaricus blazei* Murril and its mechanism of action. J. Nutr. *131*, 1409–1413.

TARDIEU, D., BAILLY, J.D., BERNARD, G. and GUERRE, P. 2007. Comparison of two extraction methods for ergosterol determination in vegetal feeds. Revue Méd. Vét. *158*, 442–446.

TEICHMANN, A., DUTTA, P.C., STAFFAS, A. and JAGERSTAD, M. 2007. Sterol and vitamin D2 concentrations in cultivated and wild grown mushrooms: Effects of UV irradiation. Food Sci. Technol -LEB *40*, 815–822.

WASSER, S.P. 2011. Current findings, future trends, and unsolved problems in studies of medicinal mushrooms. Appl. Microbiol. Biot. *89*, 1323–1332.

YOUNG, J.C. 1995. Microwave-assisted extraction of the fungal metabolites ergosterol and total fatty acids. J. Agric. Food Chem. *43*, 2904–2910.

YUAN, J.P., WANG, J.H., LIU, X., KUANG, H.C. and ZHAO, S.Y. 2007. Simultaneous determination of free ergosterol and ergosteryl esters in *Cordyceps sinensis* by HPLC. Food Chem. *105*, 1755–1759.

ZOU, X. 2005. Effects of Zn supplementation on the growth, amino acid composition, polysaccharide yields and anti-tumor activity of *Agaricus brasiliensis*. World J. Microb Biot. *21*, 261–264.

ZOU, X. 2006. Fed-batch fermentation for hyperproduction of polysaccharide and ergosterol by medicinal mushroom *Agaricus brasiliensis*. Process Biochem. *41*, 970–974.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. HPLC-PDA chromatograms obtained to quantify ergosterol in vegetal substrates extracted after submerged fermentation (SF) and solid-state fermentation (SSF) with *Agaricus brasiliensis*