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Research Paper

Optimizing a culture medium for biomass and phenolic compounds production using *Ganoderma lucidum*

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Abstract

The present work was aimed at optimizing a culture medium for biomass production and phenolic compounds by using *Ganoderma lucidum*. The culture was optimized in two stages; a Plackett-Burman design was used in the first one for identifying key components in the medium and a central composite design was used in the second one for optimizing their concentration. Both responses (biomass and phenolic compounds) were simultaneously optimized by the latter methodology regarding desirability, and the optimal concentrations obtained were 50.00 g/L sucrose, 13.29 g/L yeast extract and 2.99 g/L olive oil. Maximum biomass production identified in these optimal conditions was 9.5 g/L and that for phenolic compounds was 0.0452 g/L, this being 100% better than that obtained in the media usually used in the laboratory. Similar patterns regarding chemical characterization and biological activity towards *Aspergillus* sp., from both fruiting body and mycelium-derived secondary metabolites and extracts obtained in the proposed medium were observed. It was shown that such statistical methodologies are useful for optimizing fermentation and, in the specific case of *G. lucidum*, optimizing processes for its production and its metabolites in submerged culture as an alternative to traditional culture.

Key words: Medium optimization, *Ganoderma lucidum*, Plackett-Burman, central composite design, phenolic compounds.

Introduction

Ganoderma lucidum is a Basidiomycetes fungus from the order Polyporales, which is widely known in traditional western medicine for its immunomodulating properties and its antitumor effects. It has been shown that this fungus is useful in treating and preventing high blood pressure, hyperglycemia, hepatitis, chronic bronchitis, asthma, heart diseases, cancer and HIV (Mei-Chun *et al.*, 2006; Muller *et al.*, 2006; Lai *et al.*, 2010; Leskosek-Cukalovic *et al.*, 2010), as well as its great effect on slowing down cell senescence and its antioxidant content (Sudheesh *et al.*, 2010). *G. lucidum*'s beneficial properties are related to a broad variety of bioactive compounds present in the fruiting body, mycelium and spores. Polysaccharides, triterpens, phenols, steroids, amino acids, nucleosides and nucleotides can be found amongst such compounds. The first two have been most studied, more than 100 compounds have been identified as having biological activity; β-1-3 and β-1-6 D-glucans are the polysaccharides having the greatest biological activity and A and B ganoderic acids in the triterpens (Russell and Paterson, 2006; Leskosek-Cukalovic *et al.*, 2010).

Phenolic compounds are widely distributed amongst living organisms, particularly in plants where they play an

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important role in response to pathogenic agents, having antibacterial and antiparasitic activity, sometimes being highly specific (Del Signore *et al.*, 1997). Such compounds have one or more aromatic rings, with one or more hydroxyl groups; they can be found as simple molecules (phenolic acids, phenylpropanoids, flavonoids) or as highly polymerized compounds (lignin, melanin, tannins) and most of their biological effects have been attributed to their antioxidant activity (Barros *et al.*, 2009). Even though their presence has been reported in many species of edible and non-edible macromycetes, such as *Amanita rubescens, Boletus edulis, Cantharellus cibarius, Fistulina hepática, Hydnum repandum, Hygrophoropsis aurantiaca, Hygrophorus agathosmus, Hygrophorus olivaceo-albus, Laccaria amethystine, Lactarius aurantiacus, Lactarius salmonicolor, Lepista inversa, Mycena haematopus, Russula caerulea, Russula cyanoxantha, Russula delica, Russula sardonia, Suillus bellini, Suillus collinitus, Suillus granulatus, Suillus luteus, Suillus mediterraneensis, Tricholoma atrosquamosum, Tricholoma equestre, Tricholoma sulphureum* and *Tricholomopsis rutilans* (Ribeiro *et al.*, 2008; Barros *et al.*, 2009; Vaz *et al.*, 2011), these compounds have been little studied in the genus *Ganoderma*. It has been reported that polyphenols are the principal antioxidant components in methanol extracts of *G. lucidum* (16.5 to 27.9 $mg.g^{1}$) (Saltarelli *et al.*, 2009) and of *G. tsugae* (24.0 to 35.6 mg/g) (Mau *et al.*, 2005), such values being similar to those reported for other macromycetes such as *Antrodia camphorata* (38.0 ± 0.7 mg/g) (Song and Yen, 2002), *Ramaria botrytis* (20.32 ± 0.7 mg/g), *Hypholoma fasciculare* (17.67 ± 0.27 $mg.g^{1}$) (Barros *et al.*, 2009) and significantly higher than those found in other species, such as *Agaricus bisporus* (4.49 ± 0.16 mg/g), *Lactarius deliciosus* (3.40 ± 0.18 mg/g) and *Cantharellus cibarius* (1.75 ± 0.50 mg/g) (Barros *et al.*, 2009).

Many *G. lucidum*-based products are available on the market in the form of tea, powders and extracts due to their beneficial human health properties (Xuanwei *et al.*, 2007). They are normally produced from the fruiting body and cultured by solid state fermentation; however, this production method has serious disadvantages, including an extensive culture time (around six months), the difficulty in controlling product quality and the culture's susceptibility to environmental changes (Chang *et al.*, 2006). Efforts have thus been directed towards producing bioactive substances in submerged fermentation, mainly by studying culture conditions and media formulated for producing biomass and metabolites such as polysaccharides and ganoderic acid (Yang and Liau, 1998; Yang *et al.*, 2000; Fang *et al.*, 2002; Tang and Zhong., 2002; Chang *et al.*, 2006; Tang *et al.*, 2010; Zhu *et al.*, 2010). However, there are no reports of studies for producing phenolic compounds in submerged culture.

The present study thus established the composition of a culture medium for producing biomass and phenolic compounds from *G. lucidum* in submerged culture. A two-stage experimental strategy was used: 1) exploratory (a Plackett-Burman design was used for identifying the medium's key components) and 2) optimization (a central composite design was applied for optimizing their concentration). A desirability function was used in the latter stage for simultaneously optimizing both responses (*i.e.* biomass and phenolic compounds).

Materials and Methods

Microorganism

The *G. lucidum* basidiocarp (fruiting body) isolates were obtained from a commercial culture in Cumaral (Meta, Colombia) for the mycelium to be produced by the optimized culture medium. The fungi was conserved by plating it in YGC agar (Oxoid®) and incubated at 24 °C for nine days; discs of this agar were then placed in distilled sterile water and kept at 4 °C.

Culture media and growth conditions

The culture medium used for inoculum reactivation and production was YGC agar (Oxoid®). The liquid fermentation medium consisted of a base solution (in g/L: 1, K_2HPO_4 Carlo Erba®; 1, KH_2PO_4 Merck®; 0.5, $MnSO_4.7H_2O$ Merck®; 0.1, chloramphenicol Sigma®), added from the substrates to be evaluated, according to the detailed experimental design described later on. The mycelium conserved at 4 °C was plated in YGC agar and incubated for nine days at 24 °C. Seven discs of this agar (6 mm diameter) were inoculated in 125 mL Erlenmeyer flasks with 35 mL liquid fermentation medium; they were incubated in a shaker at 120 rpm for 10 days at 24 °C. All experiments were done in triplicate.

Analytical methods

Biomass production was measured as dry mass by membrane filtration and then dried in a convection oven for 96 hours at 60 °C. The extracellular phenolic compounds (EPC) were quantified by treating the resulting fermentation medium with 50% (v/v), final concentration, trichloroacetic acid (TCA) for precipitating proteins. This mix was then spun and the supernatant obtained; pH was adjusted to 3.5 ± 0.5 and two 1.5 mL aliquots were extracted. Polyvinyl-polypyrrolidone (PVPP) was added to one of these aliquots at 20% (p/v) final concentration; this was vigorously mixed for 30 seconds and then spun at 4 °C for 10 min at 12000 rpm. The obtained supernatant was transferred to another tube and this procedure was repeated twice more. Total phenolic compounds concentration was determined in both TCA and PVPP treated extracts and extracts just treated with TCA, using the method reported by Cliffe *et al.* (1994). The reaction was kept for 30 min at room temperature before starting to read absorbance at 775 nm wavelength. Total phenolic compounds concentra-

tion was calculated as being the difference between the concentration of extracts without PVPP and PVPP-treated extracts. The standard curve for this reaction was prepared using gallic acid. The evaluated extracts were kept at -20 °C from the time of being collected until being analyzed.

Experimental design

Selecting significant variables for a Plackett-Burman design (PBD)

PBD was initially used for evaluating the relative importance of nine parameters in *G. lucidum* biomass production in submerged culture. The following were evaluated: three carbon sources (glucose, sucrose and lactose), two organic nitrogen sources (yeast extract and peptone), an inorganic nitrogen source (ammonium chloride), adding olive oil and thiamine to the culture medium and initial pH as culture condition. Table 1 gives the experimental design, along with the variables and levels which were evaluated; each variable's effects on biomass production were estimated as well as the difference between averages for high (+) and low (-) measurements (23). Statgraphics Centurion 15.2 software was used for both design and statistical analysis, multiple regression and ANOVA.

Optimizing a central composite design (CCD)

A CCD was used for identifying optimum levels for the significant variables selected by PBD (Table 2) to obtain maximum biomass production (Y_1) and maximum EPC concentration (Y_2). The design consisted of eight factorial points, six axial points (star) and six central points in two blocks for a total of 20 experiments. The quadratic model for predicting the optimum point was expressed by the following equation:

$$Y = b_0 + \sum b_i X_i + \sum b_{ii} X_i^2 + \sum b_{ij} X_i X_j \qquad (1)$$

where Y was the response variable, b the model's regression coefficient and X the independent variables' coded levels.

Table 1 - Experimental variables at different levels in Plackett-Burman design for *G. lucidum* biomass production.

Code	Variable	Units	Experimental levels	
			Lower (-)	Higher (+)
A	Glucose	g/L	10	20
B	Sucrose	g/L	8	16
C	Lactose	g/L	10	20
D	Yeast extract	g/L	2	5
E	Peptone	g/L	2	5
F	Ammonium chloride	g/L	2	5
G	Olive oil	% (v/v)	0	3
H	Initial pH	-	5.5	6.5
I	Thiamine	mg/L	0	50

Table 2 - Independent variable levels tested for the central composite design.

Code	Variable	Units	Experimental levels				
			-1.5	-1	0	+1	+1.5
X_1	Olive oil	% (v/v)	0	1	3	5	6
X_2	Yeast extract	g/L	3	6	12	18	21
X_3	Sucrose	g/L	25	30	40	50	55

A desirability function (Eq. (2)) was used after the polynomial equations related to cell growth and EPC had been generated for selecting the most relevant factors for the response of interest in a combined response. This function was defined as being a shared function having two limiting values (minimum and maximum desirability), where values less than the established minimum were assumed to be 0, whilst values exceeding the established maximum were assumed to be 1.

$$\begin{cases} 0, & y \le y_{\min} \\ d = \dfrac{y - y_{\min}}{y_{\max} - y_{\min}}, & y_{\min} \le y \le y_{\max} \\ 1, & y \ge y_{\max} \end{cases} \qquad (2)$$

The values from the desirability functions for each replicate point, in each response evaluated (biomass and EPC) were obtained for generating a combined response; the geometric mean of both values were then calculated by assigning 3 and 5 coefficients of importance, respectively (Eq. (3)), thereby obtaining an average desirability index (D). The theoretical values used for adjusting the desirability function were assigned in line with the pertinent literature and experimental results; 18.7 g/L was thus determined for biomass, since this value was the maximum for biomass reported in optimizing biomass production using *G. lucidum* (Chang *et al.*, 2006) and the greatest experimental value obtained was used in the same design for EPC.

$$D_p = (d_b^3 \times d_c^5)^{1/8} \qquad (3)$$

Statgraphics Centurion 15.2 software was used for all design, statistical analysis, multiple regression and ANOVA operations.

Chemical characterization of biomass and culture medium extracts

Mature *G. lucidum* fruiting bodies obtained from the commercial culture and mycelium produced by the optimized culture medium were freeze-dried prior to solid-liquid extraction fractionation with petroleum ether (benzene), dichloromethane (CH_2Cl_2) and ethyl acetate (EtOAc). Each fraction was concentrated by vacuum evaporation after 96 h extraction. Concentrated fractions were then dissolved in the same solvent (for chemical characterization) or DMSO (for antifungal activity assay). The re-

maining mycelium production culture medium was extracted by liquid-liquid fractionation in the same conditions as for fruiting bodies and mycelium.

The fractions were characterized by thin layer chromatography on silica gel plates (Alugram Nano-Sil G/UV_{254} – Macherey-Nagel). The separated compounds were detected by vanillin staining (phenolics and steroids), antimony III chloride–$SbCl_3$ (terpenoids), diphenylboric acid-2-aminoethyl ester–Neu reagent (flavonoids) and potassium hydroxide–KOH (coumarins), hydroxylamine-FeCl3 (lactones) and 2, 4-dinitrophenylhydrazine-2,4 DNPH (ketones) (Merck, 1980; Jork *et al.*, 1990).

Biomass and culture medium extract antifungal activity assay

The biomass and culture medium extracts' antifungal activity regarding two clinical *Aspergillus* sp. isolates (strains 1 and 10) was evaluated by disk diffusion test following National Committee for Clinical Laboratory Standards guidelines (2003). Whatman grade No. 1 filter paper disks (6 mm diameter) were activated with 100 µg of each extract or 25 µg terbinafine as positive control for inhibiting fungal growth.

Results and Discussion

Plackett-Burman design

PBD methodology is a powerful and useful tool which is used for the rapid screening of key variables in a multivariable system; even though it does not determine exact amounts, it does provide important information about each factor by using very few experiments (Plackett and Burman, 1946; De Souza *et al.*, 2008; Prasad *et al.*, 2010). A 12 experiment PBD was used during the first optimization phase for evaluating the effect of nine variables (glucose, sucrose, lactose, yeast extract, peptone, ammonium chloride, olive oil, thiamine and initial pH) on biomass production from *G. lucidum*. The variables' interaction on two levels in PBD has revealed that yeast extract ($p = 0.027$) and olive oil ($p = 0.006$) significantly affect *G. lucidum* growth (Table 3).

Similar results have been obtained by other authors; a positive relationship has been found between adding olive oil and biomass production using *G. lucidum* and when using *Cordyceps militaris* (Park *et al.*, 2002). The olive oil is formed by several fatty acids, such as oleic, linoleic and linolenic acid, their main component being oleic acid (55%-83%). Song and Yen (2002), have proposed that oleic acid is able to directly incorporate itself into fungi cell membrane, increasing its fluidity and thereby facilitating the entry of other substrates present in culture medium. On the other hand, yeast extract is a vitamin B complex source (growth factors), amino acids, peptides and some carbohydrates, supporting effective cell development. Similar results have been obtained by Chang *et al.* (2006), whom found that yeast extract and olive oil are two substrates significantly influencing biomass development when using *G. lucidum*. Fang *et al.* (2002), have also reported that adding

Table 3 - Plackett Burman design results. The medium components and initial pH significance on *G. lucidum* biomass production are showed after 10 fermentation days.

Exp Nº	Variable code									Biomass* (g/L)
	A	B	C	D	E	F	G	H	I	
1	+	+	-	+	+	-	+	-	-	12.6 ± 0.4
2	+	-	-	-	+	+	+	-	+	7.3 ± 0.6
3	+	+	-	+	-	-	-	+	+	4.3 ± 0.7
4	+	+	+	-	+	+	-	+	-	2.6 ± 0.2
5	-	-	-	+	+	+	-	+	+	4.8 ± 0.1
6	-	-	-	-	-	-	-	-	-	2.3 ± 0.3
7	-	+	+	+	-	+	+	-	+	9.6 ± 1.4
8	-	+	-	-	-	+	+	+	-	7.8 ± 0.6
9	+	-	+	-	-	-	+	+	+	9.7 ± 1.6
10	+	-	+	+	-	+	-	-	-	5.6 ± 0.1
11	-	-	+	+	+	-	+	+	-	12.1 ± 1.1
12	-	+	+	-	+	-	-	-	+	3.7 ± 0.4
Effect	0.170	0.090	0.160	1.330	0.270	-0.770	2.960	0.220	-0.110	
F-value	0.604	0.160	0.542	36.209	1.475	12.150	178.739	0.968	0.244	
P-value	0.519	0.728	0.538	0.027[a]	0.349	0.073	0.006[a]	0.429	0.670	

*Results shown are average of three replicate experiments.
[a]Significant at 95% confidence.

organic nitrogen sources significantly promotes greater fungal growth during liquid fermentation, suggesting that some amino acids cannot be synthetized from inorganic nitrogen sources.

Regarding carbon source, no significant effect was detected for any of the three sources evaluated: glucose ($p = 0.5179$), sucrose ($p = 0.728$) or lactose ($p = 0.538$). Contradictory reports about this are found in the literature, since it has been shown that sucrose (in brown sugar form) has a stimulating effect on biomass production when using *G. lucidum* (Chang *et al.*, 2006), whilst other authors, such as Tang and Zhong (2002), have found that lactose was the best carbon source for biomass and ganoderic acid production by the *G. lucidum* CCGMC 5.616 strain and that sucrose was the carbohydrate which least promoted biomass production. Bearing the foregoing in mind, the decision to use sucrose as carbon source for later optimization was based on the results obtained in this work, since PBD did not detect differences or negative effects for the sugars evaluated regarding biomass production and/or the economic aspect.

Central composite design

A full 2^3 factorial design (eight factorial points) was used in the second optimization phase, increased by six axial points (star) and six central point replicates, giving a total of 20 experiments, for optimizing the concentration of olive oil (X_1), yeast extract (X_2) and sucrose (X_3) in the culture medium. Table 4 shows the results of these experiments, as well as the values predicted by the second order model for the three response variables (Y_1, biomass; Y_2, CEF and Y_3, D).

Table 5 presents the corresponding analysis of variance (ANOVA) results. The coefficient of determination (R^2) value provided a measure of how the variability in values observed in the response variables could be explained by the experimental factors and their interactions. So, the model's R^2 (Table 5) (0.879 for Y_1, 0.807 for Y_2 and 0.855 for Y_3) indicated that the model could explain 87.9%, 80.7% and 85.5%, respectively, of total variation in each case. P values (0.0047 for Y_1, 0.0457 for Y_2 and 0.0163 for Y_3) showed that the model represented a good prediction for the experimental results. When each response was separately analyzed, it was observed that olive oil had a significant influence on both lineal ($p = 0.0013$) and quadratics terms ($p = 0.0004$) regarding *G. lucidum* growth (Y_1). EPC concentration (Y_2) was seen to be influenced by yeast extract ($p = 0.0275$, lineal term, $p = 0.0148$ quadratic term). Regarding the combined response of biomass and EPC (D),

Table 4 - Central composite design results. The coded values of independent variables and obtained and predicted values for the three response variables are showed. The biomass and EPC responses were determined after 10 fermentation days.

Run	Independent variables (in coded value)			Response variables					
	X_1	X_2	X_3	Biomass (g/L)		EPC (g/L)		D	
				Obtained value	Predicted value	Obtained value	Predicted value	Obtained value	Predicted value
1	+1	-1	+1	9.0 ± 2.1	9.28	0.0122 ± 0.002	0.0200	0.289	0.374
2	+1	+1	-1	13.1 ± 0.5	11.86	0.0388 ± 0.003	0.0377	0.683	0.642
3	-1	+1	+1	5.2 ± 0.8	5.00	0.0576 ± 0.026	0.0490	0.618	0.530
4	+1	-1	-1	7.0 ± 1.7	7.09	0.0066 ± 0.006	0.0141	0.178	0.235
5	-1	+1	-1	6.9 ± 0.4	6.58	0.0382 ± 0.084	0.0293	0.532	0.416
6	0	0	0	10.1 ± 1.8	10.98	0.0410 ± 0.021	0.0379	0.641	0.635
7	+1	+1	+1	11.2 ± 0.7	10.79	0.0413 ± 0.018	0.0370	0.669	0.600
8	-1	-1	+1	5.7 ± 1.1	6.89	0.0362 ± 0.007	0.0361	0.479	0.489
9	-1	-1	-1	4.9 ± 0.1	5.20	0.0067 ± 0.002	0.0098	0.158	0.195
10	0	0	0	11.2 ± 2.4	10.98	0.0333 ± 0.012	0.0379	0.585	0.635
11	0	0	0	11.51.0	10.98	0.0299 ± 0.011	0.0379	0.553	0.635
12	0	-1.5	0	8.3 ± 1.2	7.05	0.0001 ± 0.000	-0.0113	0.014	-0.126
13	+1.5	0	0	5.2 ± 0.5	5.97	0.0107 ± 0.005	0.0048	0.216	0.180
14	-1.5	0	0	0.9 ± 0.6	0.21	0.0001 ± 0.000	0.0105	0.006	0.097
15	0	0	0	10.9 ± 2.2	8.75	0.0323 ± 0.005	0.0221	0.569	0.394
16	0	0	0	6.6 ± 2.5	8.75	0.0239 ± 0.004	0.0221	0.390	0.394
17	0	0	+1.5	8.8 ± 1.2	8.18	0.0419 ± 0.003	0.0461	0.618	0.645
18	0	+1.5	0	7.9 ± 1.0	9.22	0.0001 ± 0.000	0.0160	0.014	0.209
19	0	0	0	9.1 ± 2.2	8.75	0.0197 ± 0.009	0.0221	0.390	0.394
20	0	0	-1.5	7.0 ± 0.4	7.72	0.0266 ± 0.005	0.0269	0.427	0.456

Table 5 - Central composite design variance analysis. The desirability was determined using importance coefficients of 3 and 5 for biomass and EPC, respectively.

Factor	Biomass (Y_1)		EPC (Y_2)		D (Y_3)	
	P-value	Estimated effect	P-value	Estimated effect	P-value	Estimated effect
X_1	0.0013[a]	1.92	0.6517	0.007	0.4652	0.112
X_2	0.1156	0.72	0.0275 [a]	0.037	0.0133 [a]	0.063
X_3	0.7215	0.15	0.0780	0.028	0.1161	-0.113
$(X_1)^2$	0.0004 [a]	-2.52	0.1397	-0.026	0.0209 [a]	-0.156
$(X_2)^2$	0.5694	-0.27	0.0148 [a]	-0.048	0.0038 [a]	0.070
$(X_3)^2$	0.4631	-0.36	0.0582	0.034	0.1194	0.047
$X_1 * X_2$	0.1360	0.85	0.5641	0.011	0.3316	-0.039
$X_1 * X_3$	0.8133	0.13	0.7676	-0.005	0.4144	-0.045
$X_2 * X_3$	0.1512	-0.81	0.8415	0.004	0.3474	0.112
Intercept	-	9.87	-	0.18	-	0.51
Model	0.0047 [a]	-	0.0457 [a]	-	0.0163 [a]	-
Lack of fit	0.6644	-	0.0734	-	0.1052	-
R^2	0.879	-	0.807	-	0.855	-
R^2 (fitted)	0.770	-	0.633	-	0.644	-

[a]Significant at 95% confidence.

the influence of yeast extract (p = 0.0133 lineal term, p = 0.0038 quadratic term) and olive oil (p = 0.0209 quadratic term) was observed. Sucrose did not have a significant influence regarding any of the three responses.

It was not surprising that the two variables significantly affecting biomass production (X_1 and X_2) in PBD (Table 3) had equal positive influence when analyzing the combined response in CCD (Table 5). It is well-known that the synthesis of phenolic compounds in most plants, fungi and bacteria happens through the shikimic acid route where they become simple glycolysis-derived carbohydrate precursors, and the pentose shikimic acid pathway which is the precursor for most aromatic compounds in the cell (Mandal *et al.*, 2010). Such synthesis could thus be promoted by a continuous carbon flow towards the cell (Ryan *et al.*, 2002). Results obtained by some other authors have suggested that increased biomass in *G. lucidum* is directly related to the production of metabolites such as ganoderic acid and polysaccharides (Fang and Zhong, 2002; Tang and Zhong, 2002; Tang *et al.*, 2010), such results being similar to those found in this study for EPCs.

The relationship between both significant variables (X_1 y X_2) and the responses variables can be visualized in the Figure 1. The surface response curvature reflected the two independent variables' degree of influence on the dependent variables: Biomass (Figure 1A), EPC (Figure 1B) and D (Figure 1C), whilst the third variable (X_3) maintained its central value. Canonic analysis of the regression equation (Eq. (4)) revealed that the optimum concentration for the three variables leading to obtaining the maximum D response was 2.99, 13.29 and 50.00 (g/L) for olive oil (X_1), yeast extract (X_2) and sucrose (X_3), respectively.

$$D = -0.226 + 0.2147X_1 + 0.1412X_2 - 0.0346X_3 - 0.0283X_1^2 - 4.3429^{-3}X_2^2 + 6.9654^{-4}X_3^2 + 3.8750^{-3}X_1X_2 - 1.9375^{-3}X_1X_2 - 7.500^{-4}X_1X_3 \quad (4)$$

A validation experiment was carried out using the model's predicted values, maximum biomass production was 9.35 g/L (the average of three repeats: 0.535 SD) and that for phenolic compounds was 0.0452 g/L(the average for three repeats: 0.0064 SD). Comparatively, the expected desirability index was 0.653 and the experimentally index obtained (using same maximum and minimum values established for DCC) was 0.662, demonstrating the effectiveness of the method. This value was 100% higher than that obtained in the growth medium usually used in the laboratory (data not shown), indicating that optimization had been satisfactory.

Biomass and culture medium extract chemical characterization and antifungal activity

Chemical characterization revealed similar metabolite patterns for mature fruiting bodies and mycelium extracts, except for coumarins and lactones (Table 6), while terpenoids were only detected in the benzene fraction in culture medium extracts. It is probable that metabolite concentration in culture medium extracts was too low to be detected by TLC staining, but was sufficient to inhibit *Aspergillus* sp. growth (Table 6). The benzene extracts did not prevent *Aspergillus* sp. isolates growth, thereby confirming *G. lucidum* bioactive compounds' polar nature (Ofodile *et al.*, 2005). Another *G. lucidum* polar compound (ganodermin) has demonstrated mycelia growth inhibition

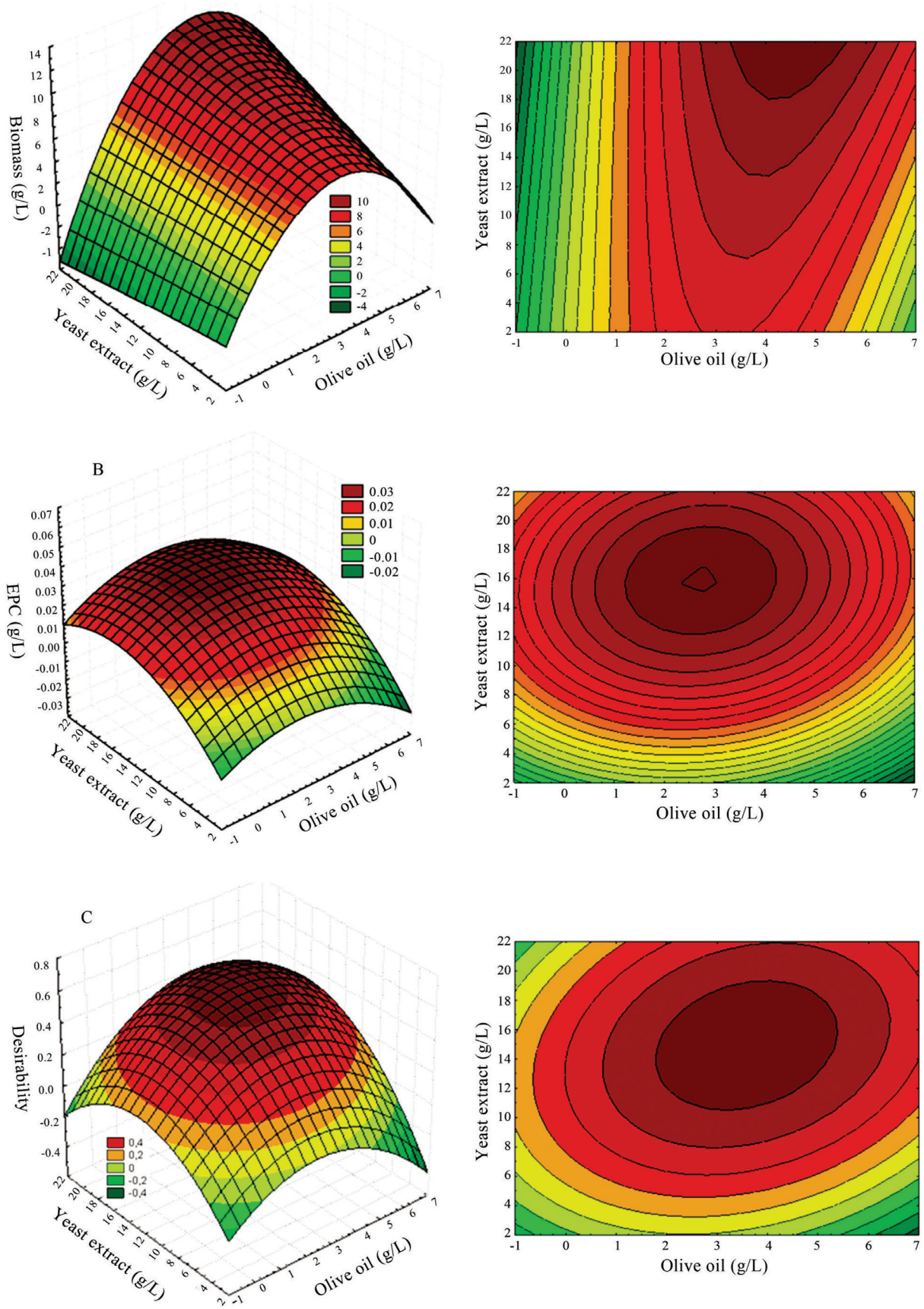

Figure 1 - 3D response surface and contours plot, showing interactive effects of olive oil (X_1) and yeast extract (X_2), sacarose (X_3) maintained at 30 g/L. Effects over: A. Biomass (g/L), B. EPC (g/L) and C. Desirability.

Table 6 - Chemical characterization and antifungal activity of biomass and culture medium extracts.

Extract source	Fraction	Chemical characterization						Antifungal activity	
		TLC stains						Inhibition growth diameter (mm)	
		Vanillin (phenolics steroids)	$SbCl_3$ (terpenoids)	Neu reagent (flavonoids)	KOH (coumarins)	Hydroxylamine $FeCl_3$ (lactones)	2 4 DNPH (ketones)	*Aspergillus* sp. (strain 1)	*Aspergillus* sp. (strain 10)
Mature fruiting bodies	Benzine	+	+	+	+	-	+	0 ± 0^{a}	0 ± 0^{a}
	CH_2Cl_2	+	+	+	+	+	+	0 ± 0^{a}	17.35 ± 2.35^{d}
	EtOAc	+	+	+	+	+	+	0 ± 0^{a}	14.41 ± 1.06^{c}
Mycelium (optimized culture medium)	Benzine	+	+	+	-	-	+	0 ± 0^{a}	0 ± 0^{a}
	CH_2Cl_2	+	+	+	-	+	+	8.50 ± 1.10^{b}	11.25 ± 1.03^{b}
	EtOAc	+	+	-	-	-	+	0 ± 0^{a}	17.52 ± 1.36^{d}
Culture medium	Benzine	+	+	-	-	-	-	0 ± 0^{a}	0 ± 0^{a}
	CH_2Cl_2	+	-	-	-	-	-	8.06 ± 0.62^{b}	13.17 ± 2.16^{c}
	EtOAc	+	-	-	-	-	-	9.75 ± 1.63^{c}	18.75 ± 1.07^{d}

of phytopathogens such as *Botrytis cinerea*, *Fusarium oxysporum* and *Physalospora piricola* (Vaz *et al.*, 2011).

Medium optimization research for *G. lucidum* has been focused on polysaccharide and terpenoid synthesis (Chang *et al.*, 2006, Fang and Zhong, 2002; Tang *et al.*, 2010), leaving apart other metabolites having diverse biological activity, such as flavonoids and ketones. Such metabolites (detected in mature fruiting bodies and mycelium extracts) have been shown to have antifungal activity regarding clinical fungal isolates (Cushnie and Lamb, 2005; Kocsis *et al.*, 2009).

Conclusion

It has been shown in this study how a culture medium for production biomass and phenolic compounds by using *Ganoderma lucidum* was optimized by using a statistical design strategy combining Plackett-Burman design, central composite design and a desirability function for optimizing both factors simultaneously. Biomass production was obtained in the optimized medium which was 100% greater than that obtained in the media usually employed in the laboratory and extracts having a biological composition and activity similar to that obtained in fruiting bodies. This showed, once more, that these methods are very useful for optimizing fermentation and, in the specific case of *G. lucidum*, optimizing processes for producing it and its metabolites in submerged culture as a sound alternative to traditional culture.

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Abbreviatures

TCA: Trichloroacetic acid.
PVPP: Polyvinyl-polypyrrolidone.
PBD: Plackett-Burman design.
CCD: Central composite design.
D: Extra average desirability index.
EPC: Extracellular phenolic compounds.

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