Carbohydrate Polymers 111 (2014) 936–946

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Influence of culture medium growth variables on *Ganoderma lucidum* exopolysaccharides structural features

Irene Fraga[a], João Coutinho[b], Rui M. Bezerra[a], Albino A. Dias[a], Guilhermina Marques[a], Fernando M. Nunes[b,*]

[a] CITAB – Centre for the Research and Technology of Agro-Environment and Biological Sciences, Universidade de Trás-os-Montes e Alto Douro, 5001-801 Vila Real, Portugal
[b] CQ-Vila Real, Chemistry Research Centre, Universidade de Trás-os-Montes e Alto Douro, Vila Real, Portugal

ARTICLE INFO

Article history:
Received 22 February 2014
Received in revised form 9 May 2014
Accepted 10 May 2014
Available online 27 May 2014

Keywords:
Ganoderma lucidum
Exopolysaccharides
Structural features
Submerged culture conditions
Central composite design
Response surface methodology

ABSTRACT

In this work the effect of carbon and nitrogen levels and initial pH of the wheat extract culture medium of submerged culture of *Ganoderma lucidum* on the amount, purity and structural features of exopolysaccharides (EPS) were studied. A low peptone level (1.65 g L^{-1}) favored mycelium biomass, EPS purity, but a higher supply of peptone (4.80 g L^{-1}) is needed for maximum EPS production. The carbohydrate composition of the EPS and structural features also changed significantly according to the different growing conditions, being observed significant differences in the (1→3)/(1→4)-Glc*p* ratio and also on the branching degree of EPS. As the biological activities of EPS are highly dependent on the polysaccharide structural features, this variability can have implications on the EPS biological activities, but can also be used advantageously to produce tailor made polysaccharides with specific applications.

1. Introduction

Ganoderma lucidum has been widely used for the general promotion of health, particularly for the prevention and treatment of several types of cancer (Wasser, 2010, chap. 78). Several studies have shown that compounds isolated from this fungus, presented cytotoxicity against cancer cells (Min, Gao, Nakamura, & Hattori, 2000) and inhibited the growth and cancer metastases (Kimura, Taniguchi, & Baba, 2002; Wasser, 2002). Therefore, *G. lucidum*, in the form of dietary supplement, can be considered as an additional therapeutic aid to cancer patients (Stanley, Harvey, Slinova, Jiang, & Sliva, 2005; Wasser, 2010). Among the bioactive compounds produced by *G. lucidum*, the polysaccharides and triterpenes have shown a range of beneficial properties such as the capacity for inducing apoptosis, inhibiting carcinogenesis and suppressing the migration of cancer cells (Stanley et al., 2005). Several research have focused on the structural features of *G. lucidum* polysaccharides, either obtained by water extraction of fruiting bodies (Bao, Wang, Dong, Fang, & Lee, 2002, Li, Fang, & Zhang, 2007; Huang, Li, Li, & Wang, 2011; Liu, Wang, Pang, Yao, & Gao, 2010; Miyasaki and Nishijima, 1981; Sone, Okuda, Wada, Kishida, & Misaki, 1985; Ye, Zhang, Zhou, et al. 2008; Ye, Zhang, Ye, et al., 2008; Ye et al., 2009; Ye, Li, Zhang, & Pan, 2010), spores (Bao, Duan, Fang, & Fang, 2001; Bao, Liu, Fang, & Li, 2001; Dong et al., 2012) and exopolysaccharides produced by this specie (Sone et al., 1985). The biological activity of the polysaccharides isolated from fruiting bodies of *G. lucidum* has been linked to their structural features, for example, Miyasaki and Nishijima (1981) found that the essential structure for the antitumor activity of a branched arabinoxyloglucan from fruiting bodies is the branched glucan core involving β-(1→3), β-(1→4) and β-(1→6) linkages. Bao et al. (2002) isolated a branched heteroglycan consisting of a α-(1→4)-D-glucose and β-(1→6)-D-galactose backbone substituted at O-6 of the glucose residues and O-2 of the galactose residues by terminal glucose, 1,6-linked glucosyl residues and terminal rhamnose (PL1) and a highly branched glucan composed of β-(1→3)-D-glucose backbone substituted at O-6 with 1,6-linked glucosyl residues (PL3) and a second heteroglycan composed by β-(1→3), β-(1→4) and β-(1→6)-D-glucose and β-(1→6)-D-mannose. All these polysaccharides showed immunoactivating properties, although PL1 was more effective. Also one glucan extracted from spores of *G. lucidum* containing (1→3), (1→3,6), (1→6) and (1→4)-linked glucose residues presented an immunostimulatory effect both *in vitro* and *in vivo* (Guo et al., 2009).

As *G. lucidum* is rare in nature, submerged culture is an advantageous and promising alternative for the production of bioactive exopolysaccharides (EPS) from this fungus (Berovic et al., 2003; Rubel, 2006). Although less studied in terms of structural features

* Corresponding author. Tel.: +351 259350242.
E-mail address: fnunes@utad.pt (F.M. Nunes).

http://dx.doi.org/10.1016/j.carbpol.2014.05.047
0144-8617/

and bioactivity, the EPS produced by *G. lucidum* have also been shown to have biological activities such as antitumor (Sone et al., 1985) and immunostimulatory (Berovic et al., 2003) activity. In the first case the EPS showed the same structural features of those from fruiting bodies, being composed of a branched β-(1→3)-glucan substituted at O-6 by single glucose units and cellobiose units at O-2 and a (1→6) linked mannan containing short chains of (1→2) linked mannose residues (Sone et al., 1985).

Nevertheless, besides the yield of EPS, as the therapeutic activity of the polysaccharides depends on their structural features such as molecular weight, chemical structure, conformation and configuration of the glycosidic linkages of polysaccharides, which in turn seem to be dependent on various biotic and abiotic factors used as biomass morphology, pH, oxygen, fermentation time, temperature, stirring and carbon and nitrogen contents, and the C/N ratio of the culture medium (Fang & Zhong, 2002; Lee, Park, Ahn, Ka, & Park, 2007; Tang & Zhong, 2002a, 2002b; Wang & McNeil, 1995; Yang & Liau, 1998), the growing conditions may affect the biological activities of the produced EPS. Also there is no consensus on the optimal growing conditions and EPS production condition for *G. lucidum*. Several authors tested different carbon sources in culture media and found that the use of glucose is advantageous in EPS yield when compared to other sugars (Elisashvili, Kachlishvili, & Wasser, 2009; Hsieh, Tseng, & Liau, 2006). Nevertheless, there is no agreement about the glucose concentration that maximizes biomass and EPS production for submerged cultures of *G. lucidum*. Also no agreement was found on the optimum pH value for biomass and EPS production, being described pH values ranging from 3.0 to 7.0 (Fang & Zhong, 2002; Kim, Park, & Yun, 2006; Lee, Lee, & Lee, 1999).

The apparent differences in the medium composition and the effect on the EPS production may be due to the interactions of the different growth variables or nonlinear effects not explored in previous studies. Also there is no knowledge on the effect of the growth variables on the EPS carbohydrate composition and structure. The ability to predict the effect of the medium culture composition on the structural features of EPS produced by *G. lucidum* is important to control and possibly to manipulate their biological activities.

In order to rationally produce high amounts of EPS and to get a better understanding on the effect of different growth variables on the EPS purity, carbohydrate composition and structural features, modern optimization techniques like response surface models seems to be the most adequate as they can describe the combined effect of several factors (Morgan, 1995). Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving and optimizing processes. The main advantage of RSM is the reduced number of experimental trials needed to evaluate multiple parameters and their interactions (Lee, Ye, Landen, & Eitenmiller, 2000; Porretta, Birzi, Ghizzoni, & Vicini, 1995). The experimental data are then used to build mathematical models, using regression methods, which if adequate, can then be analyzed using various optimization techniques to determine the optimum conditions for the process.

So, the main objectives of this study were to evaluate the level-response relationships of carbon (glucose), nitrogen (peptone) and pH in order to predict its linear and quadratic effects and interactions on mycelium biomass and EPS production, purity, carbohydrates composition, and structural features obtained from *G. lucidum* in submerged cultures using a central composite design (CCD) and the optimization of the production using response surface methodology (RSM).

2. Materials and methods

2.1. Microorganism, maintenance and pre-culture conditions

The fungus *G. lucidum* (Curtis) P. Karst., strain UF 20706 was isolated from basidiome tissue and maintained at 4 °C on potato dextrose agar (PDA) slopes by periodic subculture at the Mycology Laboratory of University of Trás-os-Montes and Alto Douro (UTAD). The mycelium was reactivated by culturing on PDA plates at 28 °C for 5 days.

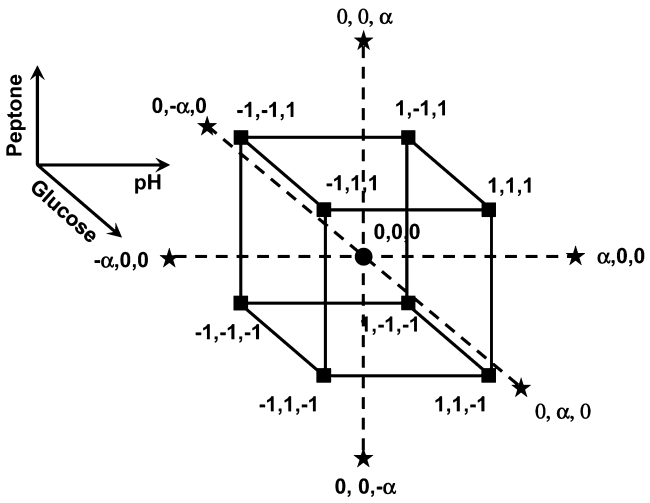
2.2. Preparation of basal medium and culture conditions

The medium was prepared by mixing 100 g of wheat grains with 1 L of distilled water. After overnight swelling, the suspension was boiled for 15 min and the aqueous extract was separated by filtration. Suspended starch was subsequently removed by centrifugation (10,000 × *g*) for 20 min at 4 °C. The medium was supplemented with 1 g L^{-1} $MgSO_4 \cdot 7H_2O$, 1.5 g L^{-1} KH_2PO_4 and different amounts of glucose and peptone as well as different initial pH values (Table 1), in a total of 15 different treatments. The

Table 1
Adimensional factors and experimental values used in the experimental design.

Treatment	Levels and coded factors			Experimental values		
	X_1	X_2	X_3	pH value	Glucose (g L^{-1})	Peptone (g L^{-1})
				X_1	X_2	X_3
Factorial points						
1	−1	−1	−1	4.0	17.5	2.5
2	−1	1	−1	4.0	35.0	2.5
3	−1	−1	1	4.0	17.5	5.0
4	−1	1	1	4.0	35.0	5.0
5	1	−1	−1	6.0	17.5	2.5
6	1	1	−1	6.0	35.0	2.5
7	1	−1	1	6.0	17.5	5.0
8	1	1	1	6.0	35.0	5.0
Central point (6 repetitions)						
9–14	0	0	0	5.0	26.25	3.75
Axial points						
15	$-\alpha$	0	0	3.32	26.25	3.75
16	α	0	0	6.68	26.25	3.75
17	0	0	$-\alpha$	5.00	26.25	1.65
18	0	0	α	5.00	26.25	5.85
19	0	$-\alpha$	0	5.00	11.55	3.75
20	0	α	0	5.00	40.95	3.75

$\alpha = (2^3)^{1/4} = 1.68$.



Scheme 1. Representation of the central composite rotatable experimental design used in this study and factor variables studied. ■ – factorial points; ● – central point; ⋆ – axial points.

medium was sterilized at 121 °C for 15 min at 1 bar. *G. lucidum* submerged cultures were carried out in 250 mL Erlenmeyer flasks with continuous stirring (150 rpm), containing 100 mL of medium at 28 °C. After 15 days of incubation, fungal biomass was separated from medium by vacuum filtration, frozen (−20 °C) and lyophilized (dry ultra-FTS systems). After lyophilization, dry weight was determined gravimetrically.

2.3. Chemical characterization of the wheat extract, glucose and peptone

The carbon and nitrogen contents of wheat extract were determined after combustion of an aliquot at 850 °C. Quantification of nitrogen was determined by chemiluminescence and quantification of the carbon by Near Infrared Detector (NIRD) in an elemental analyzer (Formac, Skalar), with oxygen as carrier gas. The content of carbon and nitrogen from peptone and glucose were determined after combustion of the solid sample at 950 °C, and the carbon content was determined by NIRD and nitrogen by thermal conductivity (Dumas) in an elemental analyzer (Primac, Skalar) with oxygen and helium as carrier gases. Briefly, wheat extract presented a C and N content of 560 mg L^{-1} and 21.5 mg L^{-1}, respectively, peptone presented a C and N content of 490 g kg^{-1} and 149 g kg^{-1}, respectively, and glucose presented a C content of 400 g kg^{-1}.

2.4. Effects of carbon, nitrogen and pH levels (experimental design)

A central composite rotatable experimental design (CCDR), was applied to evaluating the linear and nonlinear effects of the growth variables and their interactions with the biomass, EPS production, EPS purity and carbohydrate composition of the EPS, which consisted of a fractional factorial, composed of a full factorial (3 factors and 2 levels = 2^3), superimposed with a star (axial) experimental design [$\alpha = (2^3)^{1/4} = 1.68$]. A rotatable design was chosen since the variance of the predicted values is a function of the distance of a point from the center of the design and not a function of the direction of the point lies from the center (Scheme 1). Treatments were six times replicated at the central point (Morgan, 1995). Table 1 presents the adimensional and the actual values of the levels used in this experimental design for the independent variables. In order to evaluate the effects of initial pH value (X_1) and initial concentrations of glucose (X_2) as carbon source, and peptone (X_3) as nitrogen source on the response of the dependent variables (Y): fungal biomass production, EPS production, EPS purity, carbohydrate composition and crude protein content.

2.5. Isolation of the crude precipitate fraction

After incubation, the crude precipitate fraction (crude ppt) was isolated from the supernatant of cultures shake-flask after separation from the mycelia biomass. Ethanol (100 mL) was added to the filtrate to obtain a final content of 75% (v/v) and was left overnight at 4 °C (Trade Raypa) to precipitate the macromolecular components. The crude ppt was recovered by centrifugation at 10,000 × *g* (Kubota 6800 Angel Rotors RA 1500) for 20 min at 4 °C. The supernatant was discarded and the crude ppt was re-suspended in an equal volume of 75% ethanol and centrifuged again as described above. This procedure was repeated twice to remove low molecular weight material. Finally, the crude ppt was re-suspended in ultra-pure water, frozen (−20 °C) and lyophilized. Subsequently, the dry weight was determined gravimetrically.

2.6. Sugar composition and exopolysaccharides content

The EPS sugars composition was determined by high-performance anion exchange chromatography (HPAEC, ICS-3000, Dionex) with pulsed amperometric detection. Briefly, to the crude ppt (3 mg), 1 mL of sulfuric acid 12 M was added and placed in an oven (Jouan) at 35 °C for 1 h, with occasional stirring. Then, 5 mL of ultrapure water were added, and samples were hydrolyzed, during 2.5 h at 100 °C (Thermobloc-Falc). At the end of the hydrolysis 0.5 mL of the internal standard solution was added (2-deoxy-D-glucose at 1 g L^{-1}). Calibration curves were constructed and used for quantification. All analysis were performed in duplicate.

The separation was performed with a CarboPac PA-20 column (150 mm × 3 mm) with a CarboPac PA20 pre-column (Dionex) using an isocratic elution with a 1.25 mM NaOH solution containing 2 mM $Ba(OH)_2$ during 20 min, followed by a gradient elution to 150 mM sodium acetate in 25 min, maintaining during 10 more minutes. The eluent was kept under nitrogen to reduce carbonate buildup and biological contamination. The injection volume was 5 μL, the flow rate was 0.3 mL min^{-1} and the column temperature was maintained at 35 °C during the run. Electrochemical detector consisted of Au working electrode, Ag/AgCl reference electrode, and Ti counter electrode. The ED cell waveform was +0.1 V from 0.00 to 0.40 s, then −2.0 V from 0.41 to 0.42 s, and a ramp −2.0 to +0.6 V from 0.42 to 0.43 s, followed by −0.1 V from 0.44 to 0.50 s (end of cycle). The integration region was from 0.2 s to 0.4 s.

EPS content was calculated by the sum of all monosaccharides, determined by HPAEC, and EPS purity was calculated by the relative amount of total sugars to that of crude ppt.

2.7. Determination of the crude protein content

Total nitrogen content of the crude ppt was determined in the acid hydrolyzed extract by chemiluminescence in an elemental analyzer (Formac, Skalar). The crude protein content was calculated by multiplying the nitrogen content by 6.25. All analysis were performed in duplicate.

2.8. Methylation analysis

The crude ppt was methylated nine times by the NaOH–DMSO method (Nunes & Coimbra, 2001). The methylated product was hydrolyzed with TFA 2M, reduced with $NaHB_4$, acetylated with 1-methylimidazole and anhydride acetic and then analyzed by GC–MS. The partially methylated alditol acetates were identified

by their fragment ions in MS and by relative times in GC, and the molar ratios were estimated from the peak areas.

2.9. FT-IR spectrum acquisition

The FT-IR spectra of EPS samples were obtained using a Golden Gate single-reflection diamond ATR system (Specac Limited, England) in a Unicam Research Series spectrometer. The spectra were recorded in absorbance mode from 4000 to 650 cm^{-1} (mid-infrared region) at a resolution of 8 cm^{-1} with the co-addition of 512 scans. The temperature of the crystal was maintained at 25 °C. After the baselines were adjusted, the spectra were normalized to 1.

2.10. Statistical analysis

Results were submitted to ANOVA, using the software Statistica (version 8), considering statistical significance of $p < 0.05$, to quantify the effect of each of the three independent factors and their interactions, according to the experimental design.

Data was also fitted to second-order polynomial equations (Eq. (1)) for each dependent Y variable, using multiple regression analysis ($p < 0.05$) through the model:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_1^2 + \beta_5 X_2^2 + \beta_5 X_3^2 + \beta_7 X_1 X_2 + \beta_8 X_1 X_3 + \beta_9 X_2 X_3 \quad (1)$$

where β_n are the regression equation coefficients and X_n the independent variables. The significance of the model equation and model terms were evaluated by F test.

The fit quality of the polynomial model equation was expressed by the coefficient of determination R^2 and adjusted R^2. In order to visualize the relationship between the responses and each factor level of the experimental design, the fitted equation was expressed as three-dimensional surface plots.

Once achieved the adjusted model, the desirability profiles were calculated using the Profiler module of Statistica 8 for Windows (Statsoft, Tulsa, USA) to set the optimum levels of the independent variables or their combination.

2.11. Principal component analysis (PCA)

PCA is one of the most often used chemometric methods for data reduction and exploratory analysis on high-dimensional data sets. PCA decomposes the original matrix into multiplication of loading (variables) and score (samples) matrices. The principal components are linear combinations of the original variables which are uncorrelated and account for the total variance of the original variables. PCA is an unsupervised method of pattern recognition in the sense that no grouping of the data has to be known before the analysis. The new sub-space defined by the principal components leads to a model that is easier to interpret than the original data set. From these results, it should be possible to highlight several structural features and correlate them to the culture medium composition. PCA analysis was performed on the correlation matrix using the factor analysis routine of Statistica 8 for Windows (Statsoft, Tulsa, USA). Cluster analysis was performed for group formation using the K-means clustering routine of Statistica 8 for Windows (Statsoft, Tulsa, USA).

3. Results and discussion

3.1. Biomass production

The biomass production of *G. lucidum* ranged from 8.5 to 24.2 g of biomass L^{-1} (Table 2) and was significantly affected by the submerged culture conditions (Table 3), notably by the glucose level

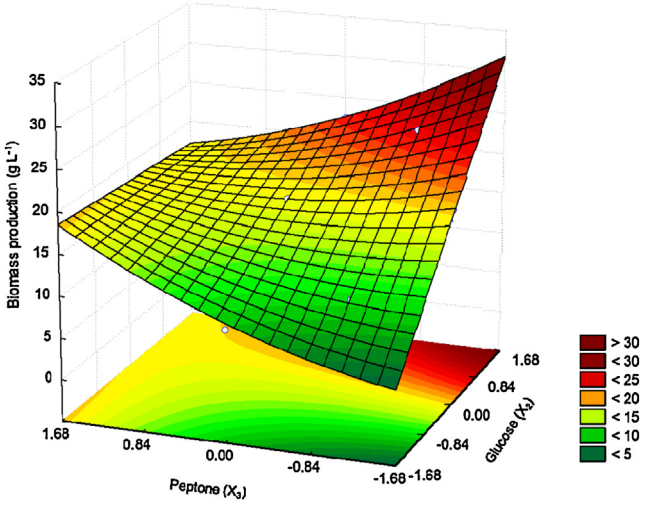


Fig. 1. Effect of glucose and peptone levels on biomass production. Factor axes are represented in coded units.

(standardized effect 11.0) but also by the pH (standardized effect −3.8) and peptone level (standardized effect −3.2). It was also observed a significant interaction between the glucose and peptone levels (standardized effect −5.5). The increase in glucose levels for low peptone levels resulted in an increase of biomass production, the inverse being observed for the highest levels of peptone (Fig. 1). Several authors also observed this expected positive effect of the carbon source on *Ganoderma* spp. (Hsieh et al., 2006; Lee et al., 2007), without noticing a depressive effect due to unfavorable osmotic pressure as suggested by Fang and Zhong (2002) above 35 g glucose L^{-1} in *G. lucidum* cultures. Huang and Liu (2008) also reported a negative effect of glucose level above 30 g L^{-1} in the cell growth of *Grifola umbelata*, that was not observed in our study.

In Table 4 there are presented the regression equations encompassing the significant effects observed by ANOVA testing (Table 3). The maximum expected biomass production calculated using the regression equation presented in Table 4 (32.3 g L^{-1}) was estimated for an initial pH value of 5.0, high levels of glucose as carbon source (40.95 g glucose L^{-1}) and low level of peptone (1.65 g L^{-1}), being equivalent to a C/N ratio of 66.8 in the medium. It is known that the pH of submerged culture is also one of the key culture conditions for mycelium biomass production by basidiomicetes (Fang & Zhong, 2002; Kim et al., 2006), since the initial pH can affect the membrane function, cell morphology and structure, the uptake of various nutrients and the synthesis of bioproducts (Shu & Lung, 2004). Similar initial pH for maximum biomass production were observed by Joo et al. (2004) and Lee et al. (2004) for cultures of *Sarcodon aspratus*, and *Grifola frondosa*, respectively. Nevertheless Huang and Liu (2008) reported that for cultures of *Grifola umbellate* a maximum yield of biomass was observed at pH 6.0, Kim et al. (2005) indicated an optimal pH of 4.0 for mycelia production of *Agrocybe cylinderacea*, and Fang and Zhong (2002) reported that a maximum biomass was obtained at an initial pH of 6.5 in *G. lucidum* cultures, in this last example they only observed a biomass production of 17.3 g L^{-1}.

3.2. Exopolysaccharide production and purity

The effect of culture condition on EPS production by *G. lucidum* was much more pronounced than that described previously for the biomass production where it was observed a 5 fold change in the obtained minimum and maximum EPS production that ranged from 115 to 531 mg L^{-1} (Table 2). Peptone level of the culture medium was the only variable showing an individual significant quadratic

Table 2
Effect of pH, carbon and nitrogen sources on different responses for the dependent variables produced in submerged culture of *Ganoderma lucidum* (Curtis) P. karst.

Treatment	C/N ratio	Production			Protein (mg g^{-1})
		Biomass (g L−1)	Crude ppt (mg L−1)	EPS (mg L−1)	
1	22.3	10.0	613	531	71.5
2	40.1	22.2	410	357	50.2
3	13.1	12.8	234	152	167
4	22.2	14.9	477	266	141
5	22.3	10.9	560	449	85.6
6	40.1	24.2	233	185	78.8
7	13.1	12.8	328	243	103
8	22.2	15.6	554	298	159
9–14[a]	22.4	16.0	624	417	101
15	22.4	12.5	337	264	87.2
16	22.4	11.2	585	517	101
17	44.8	18.9	171	115	27.2
18	15.7	16.4	572	272	156
19	12.1	8.5	327	193	130
20	32.4	22.4	743	512	79.2

[a] Central point (6 repetitions).

Table 3
Results from statistical analysis ANOVA of the effects of the independent factors on the each dependent variables.

Factor	pH (X_1)		Glucose (X_2)		Peptone (X_3)		X_1 * X_2	X_1 * X_3	X_2 * X_3
Parameter	*L*	*Q*	*L*	*Q*	*L*	*Q*	*L*	*L*	*L*
Biomass	0.769	**<0.01**	**<0.001**	0.846	**<0.01**	0.075	0.624	0.580	**<0.001**
Crude ppt	0.349	0.054	0.124	0.210	0.249	**<0.05**	0.617	0.190	**<0.05**
EPS production	0.307	0.643	0.347	0.277	0.299	**<0.01**	0.486	0.116	**<0.05**
EPS purity	0.373	**<0.001**	0.280	0.946	**<0.001**	**<0.05**	0.179	**<0.05**	**<0.05**
Protein content	0.773	0.946	0.213	0.459	**<0.001**	0.788	0.085	0.110	0.258
Carbohydrate composition									
Fucose	**<0.05**	**<0.05**	0.247	0.730	**<0.05**	0.547	0.228	0.068	0.087
Galactose	**<0.01**	**<0.01**	**<0.05**	0.204	**<0.001**	**<0.05**	0.145	**<0.01**	0.103
Glucose	**<0.01**	**<0.01**	0.081	0.593	**<0.001**	0.494	0.174	**<0.01**	**<0.05**
Mannose	**<0.05**	0.090	0.730	0.535	0.392	0.153	0.889	0.541	0.108
Structural features									
1→2-Glc*p*	0.057	**<0.05**	**<0.05**	0.061	0.137	0.066	0.530	0.319	0.185
1→3,4-Glc*p*	0.221	**<0.05**	0.511	**<0.05**	0.221	0.206	0.895	0.319	0.646
1→3,2-Glc*p*	0.833	0.245	**<0.05**	0.195	0.430	0.356	0.783	0.548	0.484
Ratio 1→3/1→4 Glc*p*	0.643	0.631	**<0.05**	0.231	0.346	0.672	0.077	**<0.05**	0.249
Substitution degree	0.065	**<0.01**	0.128	**<0.001**	**<0.05**	0.105	0.192	0.315	0.353

X_1 – pH value, X_2 – glucose (C source), X_3 – peptone (N source), *L* – linear model, *Q* – quadratic model, X_1 * X_2; X_1 * X_3; X_2 * X_3 – interactions.

effect (standardized effect −4.2), although being also observed a significant interaction between the peptone and glucose levels (standardized effect 3.0; Table 3). As it can be observed in Fig. 2A, the highest EPS production were always obtained for intermediate levels of peptone, while both high and low levels of peptone had a depressive effect on EPS production. The estimated maximum EPS production of 448.6 mg L^{-1} (Table 4) is obtained for a C/N ratio of 26.2 in the medium, much lower than for biomass production, corresponding to an application of 4.80 g peptone L^{-1} and 40.95 g glucose L^{-1}. These results show that the culture medium conditions for maximum EPS production are different from that needed for biomass production.

The initial pH value of the culture medium in the range 3.32 and 6.68 did not have a significant effect on EPS production, nevertheless Hwang, Kim, Xu, Choi, and Yun (2003) referred a pH 5.0 for optimum EPS production by *G. lucidum*, although other authors described different pH values like 3.0 (Tang, Zhang, & Zhong, 2009) and 3.5 (Fang & Zhong, 2002; Papinutti, 2010) for the same fungus, and Kim et al. (2006) indicate a pH 6.0 for optimum EPS production. These controversial findings are likely to be resultant from different morphology and physiology of the *G. lucidum* strains.

Although industrially the yield of EPS is of economic importance, the purity of the EPS is also of great concern, as during EPS isolation by ethanol precipitation there is likely to occur also pro-

Table 4
Regression equation coefficients for response parameters of *Ganoderma lucidum* (Curtis) P. Karst.

Parameter	Equation[a]	R^2	R^2 Adj.
Biomass production	$Y = 16.41 - 1.414 * X_1^2 + 3.956 * X_2 - 1.146 * X_3 - 2.583 * X_2 * X_3$	0.924	0.903
Crude ppt	$Y = 552.2 - 84.75 * X_3^2 + 124.9 * X_2 * X_3$	0.403	0.332
EPS production	$Y = 394.3 - 75.67 * X_3^2 + 75.88 * X_2 * X_3$	0.397	0.325
EPS purity	$Y = 665.3 + 68.42 * X_1^2 - 86.46 * X_3 - 24.150 * X_3^2 + 26.514 * X_1 * X_3 - 36.350 * X_2 * X_3$	0.824	0.762
Protein content	$Y = 102.0 + 36.80 * X_3$	0.746	0.733
Galactose	$Y = 65.35 - 19.07 * X_1 + 15.04 * X_1^2 + 10.65 * X_2 + 29.03 * X_3 + 8.168 * X_3^2 - 26.78 * X_1 * X_3$	0.865	0.787
Glucose	$Y = 865.2 + 31.97 * X_1 - 25.38 * X_1^2 - 35.31 * X_3 + 33.15 * X_1 * X_3 - 20.33 * X_2 * X_3$	0.815	0.748
Subst. degree 1→*x*,*y* Glc*p*	$Y = 16.63 - 1.503 * X_1^2 - 1.949 * X_2^2 - 0.955 * X_3$	0.593	0.517

[a] Independent variables are expressed in coded units (−1.68 to 1.68); 1→*x*,*y* *x* = 2 or 3 or 4 or 6 and *y* = 2 or 3 or 6.

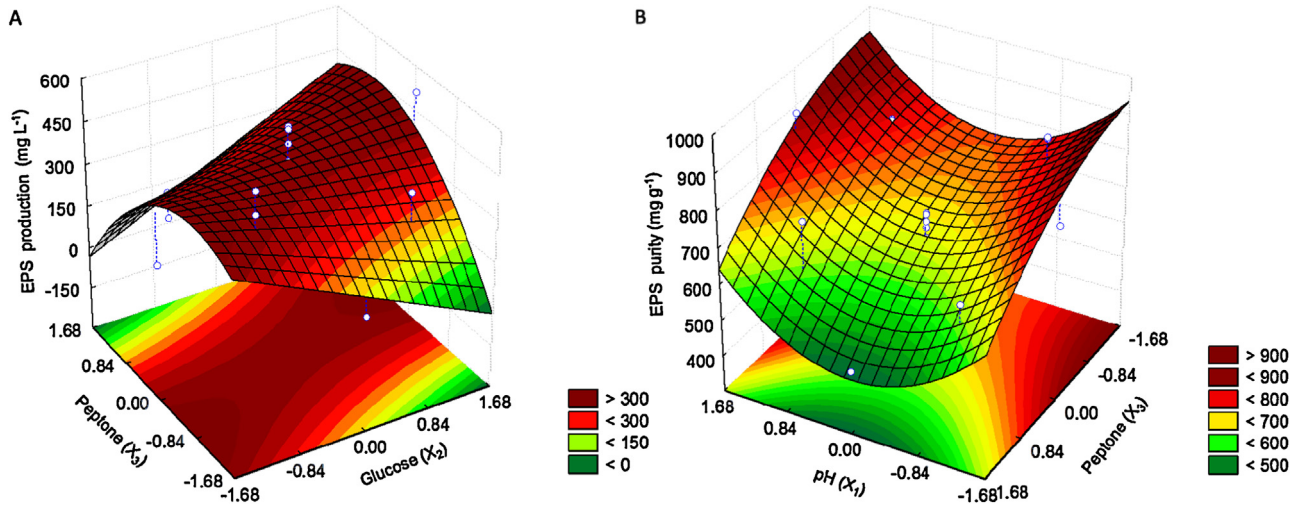


Fig. 2. Effect of glucose and peptone levels on (A) EPS production at an initial pH 5.0 and (B) effect of pH and peptone on the EPS purity by *Ganoderma lucidum*. Factor axes are represented in coded units.

tein precipitation that will affect the purity of the EPS extract, and probably also their biological activity. The culture medium conditions needed to obtain the maximum EPS purity are not the same that are needed for the maximum EPS production, as the estimated maximum EPS purity (Fig. 2B), 1113 mg g^{-1} (95% confidence interval: 989–1236 mg g^{-1}), was obtained for the same conditions of peptone and glucose indicated for the biomass production (1.65 and 11.65 g L^{-1}), at the lowest initial pH (3.32). In fact for the EPS purity a significant effects of initial pH and peptone (linear and quadratic) was observed, being also observed a significant interaction between glucose and peptone and also interaction between pH and peptone (Table 3).

3.3. Carbohydrate composition and structural features of exopolysaccharides

Glucose was the main sugar present in *G. lucidum* EPS (69–93%, Table 5) followed by galactose (4–19%) and much lower amounts of mannose (2–8%) and fucose (1–5%). The relative abundance of glucose in EPS was significantly influenced by the culture medium conditions with the pH being the culture medium factor with a higher influence (standardized linear effect of 6.5 and standardized quadratic effect of −5.4) as well it was observed a significant interaction with peptone level (standardized effect of 5.2, Fig. 3A).

The peptone level of the medium culture also showed a high significant effect on the relative abundance of glucose (standardized linear effect −7.2). So for high peptone levels with increasing pH values the relative abundance of glucose increases, the reversed being observed for low peptone levels (Fig. 2A). The maximum relative abundance of glucose in the EPS estimated by regression (Table 4) is 984 mg g^{-1} being obtained at initial pH value of 4.16, maximum level of glucose in the culture medium and for the minimum level of peptone (1.65 g L^{-1}), corresponding to C/N ratio of 66.8. These findings are consistent with the results obtained by Kim et al. (2006) for the EPS of *G. lucidum*, where glucose content increased when pH was kept constant at 6.0, when compared with lower pH values.

In Fig. 3B it is presented the effect of peptone and pH on the galactose content of the EPS. A strong positive effect of peptone is evident for low initial pH values, in opposition to that observed for glucose, and the effect of initial pH depends on peptone level: a depressive effect of pH at high levels of peptone, and a positive, but moderate, effect of initial pH at low levels of peptone, similar to that observed for glucose. The maximum galactose content of the EPS estimated by regression (Table 4) was 330 mg g^{-1}, obtained at the lowest initial pH value (3.32) and for the maximum levels of peptone (5.85 g L^{-1}) and glucose (40.95 g L^{-1}), corresponding to C/N ratio of 22.2, lower than the one indicated for the glucose content.

These results show that the culture medium conditions affect significantly the carbohydrate composition of the EPS, and these can be due to different structural details of a single polysaccharide or due to the varying abundance of different polysaccharides produced. Whatever the reason this can have a direct impact on the biological activity of the extracts obtained. So in order to ascertain if the medium culture conditions also affect the structural details of the EPS produced by *G. lucidum*, these were determined by methylation analysis (Table 6). The linkages present in higher abundance are those corresponding to the glucose residues mainly linked by (1→4) linkages with an abundance ranging from 45 to 66 molar%, being also present significant amounts of (1→3) (5–20%) and lower amounts of (1→2) linked glucose residues. There were also present significant amounts of branched glucose residues, mainly (1→4,6), (1→3,4) and (1→2,4) glucose residues and lower amounts of (1→2,3) glucose residues. The high abundance of terminally linked glucose residues is consistent with the branching abundance observed. The relative abundance of all substituted glucose residues ranged from 10% to 20%. The galactose residues were mainly present as (1→6) linked galactose residues and terminally linked. The structural features of the EPS obtained in this study were significantly different from those described previously by Li et al. (2007) and Sone et al. (1985) that were also different between these two studies. EPS described by Sone et al. (1985) were composed by branched β-(1→3)-glucan substituted at O-6 by single glucose units and cellobiose units at O2 and a (1→6) linked mannan containing short chains of (1→2) linked mannose residues on the other hand the EPS described by Li et al. (2007) were mainly composed by a branched (1→4)-linked galactan substituted at O-6 position of the galactose residues by single L-rhamnosyl, L-arabinosyl residues and 1,6-linked α-D-mannopyranosyl and 1,4-linked β-D-glucopyranosyl residues. Nevertheless the EPS obtained in this study are very similar to those water extractable polysaccharides present in fruiting bodies of *G. lucidum* described by Bao et al. (2002) and Miyasaki and Nishijima (1981), where the isolated and purified polysaccharides were composed by a substantially higher abundance of (1→4)-linked glucose residues when compared to the (1→3)-linked glucose residues. These differences between the

Table 5
Effect of pH, carbon and nitrogen sources on carbohydrate composition and sugar purity produced by *Ganoderma lucidum* (Curtis) P. Karst.

Treatment	Carbohydrate composition				EPS purity (mg g^{-1} crude ppt)
	Fucose (mg g^{-1} EPS)	Galactose (mg g^{-1} EPS)	Glucose (mg g^{-1} EPS)	Mannose (mg g^{-1} EPS)	
1	30.9	50.2	860	58.9	866
2	25.2	61.7	876	37.4	870
3	38.4	157	749	55.7	647
4	45.6	195	694	65.8	557
5	23.3	54.9	863	58.5	802
6	26.3	87.7	849	36.8	796
7	18.6	46.1	895	39.9	741
8	32.7	123	789	55.8	538
9–14[a]	21.3	62.0	869	48.1	696
15	31.7	128	754	86.1	784
16	20.3	64.0	884	31.9	883
17	14.0	37.9	930	18.0	670
18	19.9	115	835	30.4	475
19	15.9	69.4	881	33.9	589
20	16.3	61.8	889	33.4	690

[a] Central point (6 repetitions).

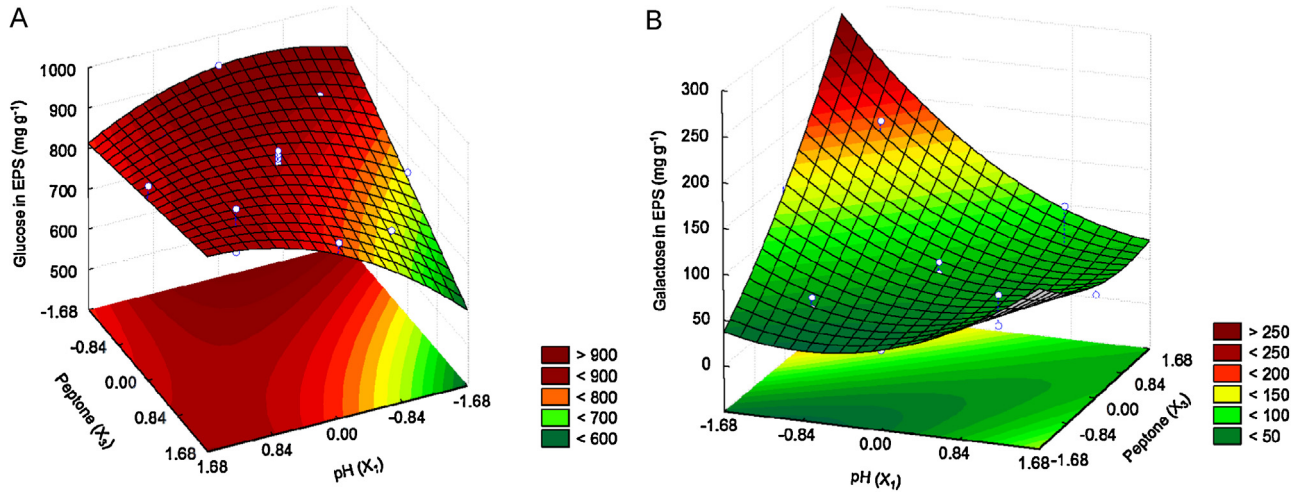


Fig. 3. Effect of peptone level and initial pH on (A) glucose content of EPS and (B) on galactose content of EPS effects at 26.25 g glucose L^{-1} produced by *Ganoderma lucidum*. Factor axes are represented in coded units.

structural features of the EPS obtained in this work and those previously described by Li et al. (2007) and Sone et al. (1985) can be due to different strains used, or due to the different growing conditions.

FTIR characterization of the different EPS obtained under the different growing conditions (exemplified in Fig. 4) did not show notable differences between them. It was possible to observe the characteristic bands of (1→4) linked glucose residues at 1041, 1153 cm^{-1}, and the small peak at 891 cm^{-1} typical of β-glucans (Gutiérrez, Prieto, & Martínez, 1996), suggesting that the EPS produced by *G. lucidum* are mainly β-glucans, as also notice for the β-(1→4) linked water soluble glucans recovered from the fruiting bodies of *G. lucidum* (Bao et al., 2002; Miyasaki & Nishijima, 1981).

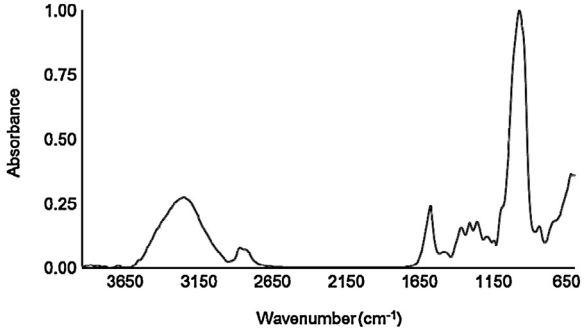


Fig. 4. FTIR spectra (4000–650 cm^{-1} region) of EPS of *Ganoderma lucidum* obtained under submerged culture conditions at the factorial point 3 (Table 1). Bands are assigned in the text.

The medium culture conditions influenced significantly the branching degree of the EPS produced by *G. lucidum*, with the glucose level presenting the higher effect (standardized quadratic effect of −7.2) followed by the pH value (standardized quadratic effect of −5.6) and with a lower effect the peptone level (standardized linear effect of −3.3). The maximum branching degree estimated of 18 molar% (Fig. 5A–C) can be obtained at medium pH value and glucose levels (5.0 and 26.25 g L^{-1}, respectively) and low peptone levels (1.65 g L^{-1}), on the other hand a lower branching degree can be obtained (5.3 molar%) by using higher or lower pH values, lower or higher glucose levels and high peptone levels.

The glucose level of the medium culture conditions also affected significantly the (1→3)/(1→4) Glc*p* ratio of the EPS (Table 3) and there was also a significant interaction between the pH value and peptone levels of the culture medium, nevertheless the regression model obtained was not good enough for predicting the influence of the medium culture conditions on this structural detail meaning that this structural feature cannot be adequately modeled by the studied variables, or a more complicated model or additional

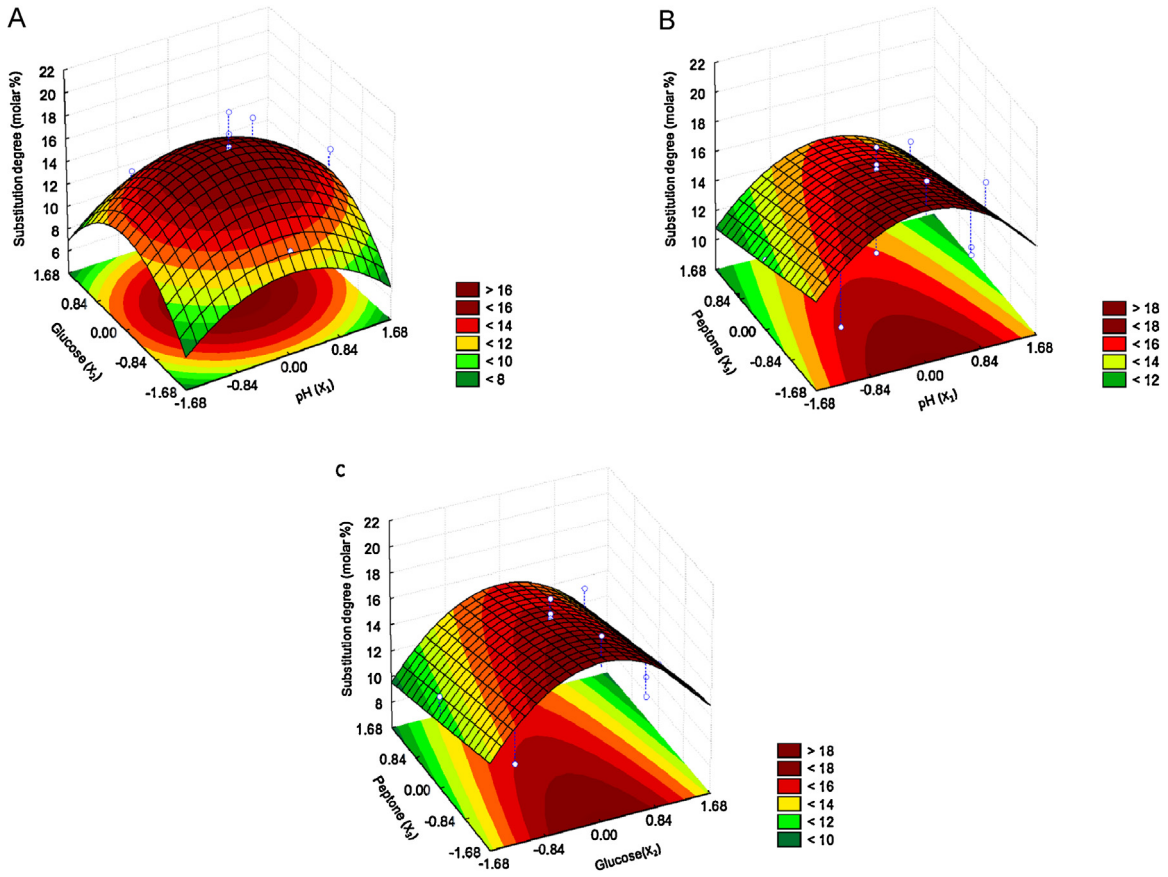


Fig. 5. Effect of pH and peptone (A), pH and glucose (B), glucose and peptone (C) on substitution degree produced by *Ganoderma lucidum*. Factor axes are represented in coded units.

factors are required to a higher fit of the experimental data (Box, Hunter, & Hunter, 1978). Other structural features of the EPS produced were also significantly influenced by the medium culture conditions, like the (1→3,4)-Glc*p*, (1→3,2)-Glc*p* and (1→2)-Glc*p* abundance, nevertheless the models obtained were also not good enough for predicting the influence of the culture medium composition on the structural details.

In order to gain a deeper insight of the internal data structure, a principal component analysis was performed on the data set. The PCA of all structural details of the EPS produced by the *G. lucidum* under different culture conditions yielded two principal components explaining more than 70% of the total variance in the original data set (Fig. 6B). The first PC, which explains 42.9% of the total variance, correlates negatively ($r < -0.70$) with the structural details (1→3)-Glc*p*, (1→3)/(1→4)-Glc*p* and T-Gal*p* and positively ($r > 0.70$) with the structural details (1→4)-Glc*p* and (1→6)-Gal*p*. The second PC, which explains 30.3% of the total variance, correlates negatively ($r < -0.70$) with the structural details relating to the branching degree of the EPS namely (1→3,4)-Glc*p*, (1→2,4)-Glc*p* and the total branching degree (1→*x*,*y*)-Glc*p* (Fig. 6B). K-mean clustering of the first and second principal component sample scores yielded three groups (ANOVA, PC1 $p < 0.00032$ and PC2 $p < 0.0024$).

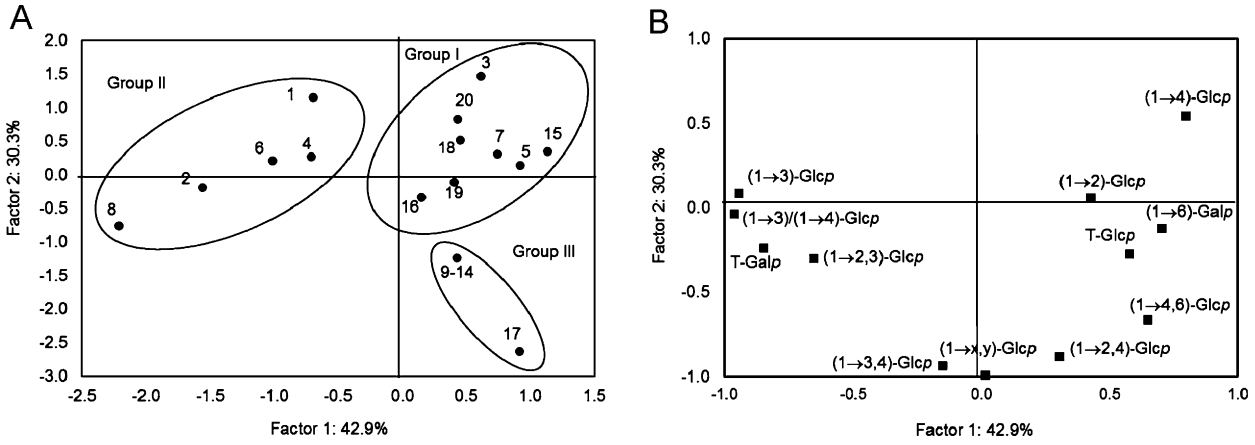


Fig. 6. Results of principal component analysis (A) score plot of the EPS samples produced by *Ganoderma lucidum* projected on space of PC1 *vs* PC2. (B) Loading plot of the structural features of EPS projected on the space PC1 *vs* PC2.

Table 6
Structure features of exopolysaccharides produced by *Ganoderma lucidum* in submerged culture.

Treatment	Linkage type and molar percent (%)										Ratio	Subst. degree
	T-Gal	T-Glc	1→2 Glc*p*	1→6 Gal*p*	1→3 Glc*p*	1→4 Glc*p*	1→3,4 Glc*p*	1→3,2 Glc*p*	1→4,2 Glc*p*	1→4,6 Glc*p*	1→3 Glc*p*/1→4 Glc*p*	1→x,y Glc*p*[b]
1	5.5	8.7	2.7	1.7	17.9	53.6	2.8	0.9	1.6	4.7	0.33	9.9
2	6.1	8.5	1.7	0.70	19.4	49.6	4.6	2.2	2.5	4.7	0.39	14.0
3	3.9	10.9	1.9	1.3	6.4	66.1	2.5	0.5	1.7	4.8	0.10	9.6
4	5.3	11.7	1.6	1.1	14.5	53.2	3.0	2.5	1.6	5.7	0.27	12.8
5	3.5	10.6	2.2	2.0	7.1	61.6	4.1	0.3	2.6	6.0	0.11	13.0
6	4.7	9.9	1.6	0.79	18.8	51.8	3.9	1.3	2.4	4.9	0.36	12.5
7	4.3	13.9	1.9	1.5	6.7	59.7	3.5	0.5	2.3	5.7	0.11	12.0
8	8.3	8.7	1.6	0.54	20.3	45.3	6.3	2.3	2.3	4.4	0.45	15.3
9–14[a]	3.8 (1.0)[c]	9.9 (1.7)	1.9 (0.21)	3.0 (1.5)	9.2 (3.7)	55.0 (4.7)	6.2 (1.2)	1.5 (0.8)	3.0 (0.9)	6.4 (1.2)	0.21 (0.06)	17.0 (1.0)
15	2.9	9.5	2.0	2.6	6.7	63.6	3.6	0.3	2.4	6.5	0.11	12.7
16	3.0	12.0	1.3	0.74	11.4	57.3	4.4	1.0	3.0	5.9	0.20	14.3
17	5.0	13.9	2.5	1.8	4.9	51.7	7.0	1.5	4.3	7.5	0.09	20.3
18	3.3	12.3	2.4	1.7	7.8	60.1	3.3	2.1	2.1	4.9	0.13	12.4
19	3.8	10.9	2.6	1.8	12.8	54.9	4.4	0.1	2.9	5.8	0.23	13.2
20	3.6	10.4	2.3	1.5	8.9	62.0	3.3	0.9	2.2	4.9	0.14	11.3

[a] Central point (6 repetitions).
[b] 1→*x*,*y*; *x* = 2 or 3 or 4 or 6 and *y* = 2 or 3 or 6.
[c] The values in parenthesis represent the standard deviation.

A first group with higher PC1 and PC2 scores containing samples 3, 5, 7, 15, 16, 18, 19 and 20 (Fig. 6A) are characterized by EPS containing higher abundances of (1→4)-Glc*p* and (1→6)-Gal*p* and lower branching degree (Fig. 6B). A second group consisting in samples 1, 2, 4, 6 and 8, show positive PC1 scores and negative PC2 scores (Fig. 6A), being characterized by EPS containing a higher abundance of (1→3)-Glc*p*, (1→3)/(1→4)-Glc*p* and T-Gal*p* structural details and also a lower branching degree (Fig. 6B). The third group presents positive PC1 and negative PC2 scores contains samples 9–14 and 17, Fig. 6A being different from the samples present in the first group, presenting a higher branching abundance (Fig. 6B). By analysis of the growing culture variables of the samples presented in each group it can be inferred that samples presented in group 2 were obtained under pH 4 or 6 with high amounts of glucose (35.5 g L^{-1}), with the exception of sample 1, with the lowest negative PC1 score, that presents a lower amount of glucose (17.5 g L^{-1}) but nevertheless a high C/N ratio (22.3). From these results it can be concluded that a higher abundance of glucose in the growth culture medium and a pH of 4 or 6 results in EPS with a higher abundance of (1→3)-linkages, (1→3)/(1→4)-Glc*p* ratio and T-Gal*p* residues. On the other hand for obtaining EPS with higher branching degree, a pH of 5 should be used using low levels of peptone in the growth culture medium (group 3). These results show that the medium culture growth variables influence decisively the structural features of the EPS produced by *G. lucidum*.

The influence of the medium culture variables on EPS structural features and EPS yield previously discussed, can be linked to changes in fungus cell morphology that are known to be affected by factors such as pH, carbon and nitrogen levels (Tang, Zhu, Li, & Li, 2007). Although the relationship between growth morphology and metabolite production by mushrooms is not well understood (Wagner, Mitchell, Sassaki, & Amazonas, 2004), one or both of two growth forms occur during the mushroom cultivation in submerged culture: the filamentous and the pellet form (Sinha et al., 2001). The fungus cell morphology has a direct impact on broth rheology that also affects mushroom metabolism due to a decrease in mass and heat transfer, oxygen uptake, carbon dioxide evolution and mixing efficiency (Hwang, Kim, Xu, Choi, & Yun, 2004; Pazouki & Panda, 2000). For example, cultures in which the mushroom grows as pellets tend to be less viscous than those in which it grows as dispersed filaments. In the first case the culture broth typically only becomes viscous at high biomass concentrations (Gibbs, Seviour, & Schmid, 2000). Additionally the influence of the medium culture conditions can also be linked to the differential activity of the α-phosphoglucomutase, a key enzyme which leads to EPS formation at the branch point between the Embden–Meyerhof–Parnas pathway at the later part of EPS biosynthesis (Tang & Zhong, 2002a, 2002b).

For several bioactive polysaccharides isolated from different mushrooms the structural features are essential for their activity, namely structural features such as β-(1→3) linkages in the main chain of the glucan and additional β-(1→6) branch points are needed for antitumor activity and β-glucans containing mainly (1→6) linkages have less activity (Wasser, 2002; Ren, Perera, & Hemar, 2012). For *G. lucidum* polysaccharides isolated from fruiting bodies, Miyasaki and Nishijima (1981) showed that the essential structure for the antitumor activity of a branched arabinoxyloglucan is the branched glucan core involving β-(1→3), β-(1→4) and β-(1→6) linkages. Bao et al. (2002) also showed that the most immunomodulating polysaccharide isolated from *G. lucidum* fruiting bodies was composed by a backbone of α-(1→4)-D-glucose and β-(1→6)-D-galactose residues substituted at O-6 of glucose residues and O-2 of galactose residues by terminal glucose, 1,6-linked glucosyl residues and terminal rhamnose. So the significant differences in structural features of the EPS produced by *G. lucidum* using different growth culture variables will have most probably

an impact on their biological activities. Nevertheless the results obtained in this work also show that the structure of the EPS can be tailored by simple manipulation of the growth culture medium variables, and this can be used advantageously when a structure bioactivity relation for the EPS from *G. lucidum* are known.

4. Conclusions

The culture medium conditions have a significant influence on the structural features and the yield of the EPS produced by *G. lucidum* under submerged culture conditions. High glucose levels of the growth culture medium and pH 4 and 6 favored the formation of EPS with a higher abundance of (1→3)-linkages and (1→3)/(1→4)-Glc*p* ratio, and low levels of peptone on the growth culture medium and pH 5 favored the formation of EPS with a higher branching degree. As the biological activities of EPS from other basidiomicetes including that of *G. lucidum* are highly dependent on the polysaccharide structural features, the variability observed in the structural features dependent on the medium culture conditions can have serious implication on the biological activities of the EPS produced. On the other hand the influence of the culture medium conditions can also be used advantageously to produce tailor made polysaccharides with specific applications.

Acknowledgments

The authors greatly acknowledge the helpful discussion with Fernanda Cabral and Alfredo Aires. We also thank André Lemos and Pedro da Silva for the technical support. We appreciate the financial support provided to the Research Unit in Vila Real (PEst-OE/QUI/UI0616/2014) and CITAB (FCOMP-01-0124-FEDER-022692) by the Foundation for Science and Technology (FCT) and COMPETE.

References

Bao, X., Duan, J., Fang, X., & Fang, J. N. (2001). Chemical modifications of the (1→3)-α-D-glucan from spores of *Ganoderma lucidum* and investigation of their physicochemical properties and immunological activity. *Carbohydrate Research*, *336*, 127–140.

Bao, X., Liu, C., Fang, J., & Li, X. (2001). Structural and immunological studies of a major polysaccharide from spores of *Ganoderma lucidum* (Fr.) Karst. *Carbohydrate Research*, *332*, 67–74.

Bao, X.-F., Wang, X.-S., Dong, Q., Fang, J.-N., & Li, X.-Y. (2002). Structural features of immunologically active polysaccharides from *Ganoderma lucidum*. *Photochemistry*, *59*, 175–181.

Berovic, M., Habijanic, J., Zore, I., Wraber, B., Hodzar, D., Boh, B., et al. (2003). Submerged cultivation of *Ganoderma lucidum* biomass and immunostimulatory effects of fungal polysaccharides. *Journal of Biotechnology*, *103*, 77–86.

Box, G. E. P., Hunter, W. G., & Hunter, J. S. (1978). *Statistics for experimenters*. New York: John Wiley and Sons.

Dong, Q., Wang, Y., Shi, L., Yao, J., Li, J., Ma, F., et al. (2012). A novel water-soluble β-D-glucan isolated from the spores of *Ganoderma lucidum*. *Carbohydrate Research*, *353*, 100–105.

Elisashvili, V. I., Kachlishvili, E. T., & Wasser, S. P. (2009). Carbon and nitrogen source effects on basidiomycetes exopolysaccharide production. *Applied Biochemistry and Microbiology*, *45*, 592–596.

Fang, Q.-H., & Zhong, J.-J. (2002). Effect of initial pH on production of ganoderic acid and polysaccharide by submerged fermentation of *Ganoderma lucidum*. *Process Biochemistry*, *37*, 769–774.

Gibbs, P. A., Seviour, R. J., & Schmid, F. (2000). Growth of filamentous fungi in submerged culture: Problems and possible solutions. *Critical Reviews in Biotechnology*, *20*, 17–48.

Guo, L., Xie, J., Ruan, Y., Zhou, L., Zhu, H., Yun, X., et al. (2009). Characterization and immunostimulatory activity of a polysaccharide from the spores of *Ganoderma lucidum*. *International Immunopharmacology*, *9*, 1175–1182.

Gutiérrez, A., Prieto, A., & Martínez, A. T. (1996). Structural characterization of extracellular polysaccharides produced by fungi from the genus *Pleurotus*. *Carbohydrate Research*, *281*, 143–154.

Hsieh, C., Tseng, M.-H., & Liu, C.-J. (2006). Production of polysaccharides from *Ganoderma lucidum* (CCRC 36041) under limitations of nutrients. *Enzyme and Microbial Technology*, *38*, 109–117.

Huang, H.-C., & Liu, Y.-C. (2008). Enhancement of polysaccharide production by optimization of culture conditions in shake flask submerged cultivation of *Grifola umbellata*. *Journal of the Chinese Institute of Chemical Engineers*, *39*, 307–311.

Huang, S. Q., Li, J. W., Li, Y. Q., & Wang, Z. (2011). Purification and structural characterization of a new water-soluble neutral polysaccharides GLP-F1 from *Ganoderma lucidum*. *International Journal of Biological Macromolecules*, *48*, 165–169.

Hwang, H. J., Kim, S. W., Xu, C. P., Choi, J. W., & Yun, J. W. (2003). Production and molecular characteristics of four groups of exopolysaccharides from submerged culture of *Phellinus gilvus*. *Journal of Applied Microbiology*, *94*, 708–719.

Hwang, H. J., Kim, S. W., Xu, C. P., Choi, J. W., & Yun, J. W. (2004). Morphological and rheological properties of the three different species of basidiomycetes *Phellinus* in submerged cultures. *Journal of Applied Microbiology*, *96*, 1296–1305.

Joo, J. H., Lim, J. M., Kim, H. O., Kim, S. W., Hwang, H. J., Choi, J. W., et al. (2004). Optimization of submerged culture conditions for exopolysaccharide production in *Sarcodon aspratus* (Berk) S.Ito TG-3. *World Journal of Microbiology and Biotechnology*, *20*, 767–773.

Kim, H. O., Lim, J. M., Joo, J. H., Kim, S. W., Hwang, H. J., Chi, J. W., et al. (2005). Optimization of submerged culture condition for the production of mycelial biomass and exopolysaccharides by *Agrocybe cylindracea*. *Bioresource Technology*, *96*, 1175–1182.

Kim, H. M., Park, M. K., & Yun, J. W. (2006). Culture pH affects exopolysaccharide production in submerged mycelial culture of *Ganoderma lucidum*. *Applied Biochemistry and Biotechnology*, *134*, 249–262.

Kimura, Y., Taniguchi, M., & Baba, K. (2002). Antitumor and antimetastatic effects on liver triterpenoid fractions of *Ganoderma lucidum*: Mechanism of action and isolation of active substance. *Anticancer Research*, *22*, 3309–3318.

Lee, K. M., Lee, S. Y., & Lee, H. Y. (1999). Bistage control of pH for improving exopolysaccharides production from mycelia of *Ganoderma lucidum* in Air-Lift Fermentor. *Journal of Bioscience and Bioengineering*, *88*, 646–650.

Lee, J., Ye, L., Landen, W. O., & Eitenmiller, R. R. (2000). Optimization of an extraction procedure for the quantification of vitamin E in tomato and broccoli using response surface methodology. *Journal of Food Composition and Analysis*, *13*, 45–57.

Lee, B. C., Bae, J. T., Pyo, H. B., Choe, T. B., Kim, S. W., Hwang, H. J., et al. (2004). Submerged culture conditions for the production of mycelia biomass and exopolysaccharides by edible basidiomycete *Grifola frondosa*. *Enzyme Microbial Technology*, *35*, 369–376.

Lee, W. Y., Park, Y., Ahn, J. K., Ka, K. H., & Park, S. Y. (2007). Factors influencing the production of endopolysaccharides and exopolysaccharides from *Ganoderma applanatum*. *Enzyme and Microbial Technology*, *40*, 249–254.

Li, Y. Q., Fang, L., & Zhang, K. C. (2007). Structure and bioactivities of a galactose rich extracellular polysaccharide from submergedly cultured *Ganoderma lucidum*. *Carbohydrate Polymers*, *68*, 323–328.

Liu, W., Wang, H. Y., Pang, X. B., Yao, W. B., & Gao, X. D. (2010). Characterization and antioxidant activity of two low-molecular-weight polysaccharides purified from the fruiting bodies of *Ganoderma lucidum*. *International Journal of Biological Macromolecules*, *46*, 451–457.

Min, B.-S., Gao, J.-J., Nakamura, N., & Hattori, M. (2000). Triterpenes from the spores of *Ganoderma lucidum* and their cytotoxicity against meth-A and LLC tumor cells. *Chemical and Pharmaceutical Bulletin*, *48*, 1026–1033.

Miyasaki, T., & Nishijima, M. (1981). Studies on fungal polysaccharides: XXVII. Structural examination of a water-soluble, anti-tumor polysaccharide of *Ganoderma lucidum*. *Chemical and Pharmaceutical Bulletin*, *29*, 3611-36-16.

Morgan, E. (1995). *Chemometrics: Experimental design (analytical chemistry by open learning)* (second ed., pp. 189–260). USA: John Wiley Ltd.

Nunes, F. M., & Coimbra, M. A. (2001). Chemical characterization of the high molecular weight material extracted with hot water from green and roasted Arabica coffee. *Journal of Agricultural and Food Chemistry*, *49*, 1773–1782.

Papinutti, L. (2010). Effects of nutrients, pH and water potential on exopolysaccharides production by a fungal strain belonging to *Ganoderma lucidum* complex. *Bioresource Technology*, *101*, 1941–1946.

Pazouki, M., & Panda, T. (2000). Understanding the morphology of fungi. *Bioprocess Engineering*, *22*, 127–143.

Porretta, S., Birzi, A., Ghizzoni, C., & Vicini, E. (1995). Effects of ultra-high hydrostatic pressure treatments on the quality of tomato juice. *Food Chemistry*, *52*, 35–41.

Ren, L., Perera, C., & Hemar, Y. (2012). Antitumor activity of mushroom polysaccharides: A review. *Food and Function*, *3*, 1118–1130.

Rubel, R. (2006). *Produção de compostos bioativos de Ganoderma lucidum por fermentação em estado sólido: avaliação da ação antitumoral, imunomoduladora e hipolipidêmica*. Curitiba, Brasil: Pós-Graduação em Processos Biotecnológicos, Universidade Federal do Paraná.

Shu, C. H., & Lung, M. Y. (2004). Effect of pH on the production and molecular weight distribution of exopolysaccharide by *Antrodia camphorata* in batch cultures. *Process Biochemistry*, *39*, 931–937.

Sinha, J., Bae, J. T., Park, J. P., Kim, K. H., Song, C. H., & Yun, J. W. (2001). Changes in morphology of *Paecilomyces japonica* and their effect on broth rheology during production of exo-biopolymers. *Applied Microbiology and Biotechnology*, *56*, 88–92.

Sone, Y., Okuda, R., Wada, N., Kishida, E., & Misaki, A. (1985). Structures and antitumor activities of the polysaccharides isolated from fruiting body and the growing culture of mycelium of *Ganoderma lucidum*. *Agricultural and Biological Chemistry*, *49*, 2641–2653.

Stanley, G., Harvey, K., Slivova, V., Jiang, J., & Sliva, D. (2005). *Ganoderma lucidum* suppresses angiogenesis through the inhibition of secretion of VEGF and TGF-b1 from prostate cancer cells. *Biochemical and Biophysical Research Communications*, *330*, 46–52.

Tang, Y. J., & Zhong, J. J. (2002). Exopolysaccharide biosynthesis and related enzyme activities of the medicinal fungus, *Ganoderma lucidum*, grown on lactose in a bioreactor. *Biotechnology Letters*, *24*, 1023–1026.

Tang, Y.-J., & Zhong, J.-J. (2002). Fed-batch fermentation of *Ganoderma lucidum* for hyperproduction of polysaccharide and ganoderic acid. *Enzyme and Microbial Technology*, *31*, 20–28.

Tang, A.-J., Zhu, L.-W., Li, H.-M., & Li, D.-S. (2007). Submerged culture of mushrooms in bioreactors – Challenges: Current state-of-the-art, and future prospects. *Food Technology and Biotechnology*, *45*, 221–229.

Tang, Y.-J., Zhang, W., & Zhong, J.-J. (2009). Performance analyses of a pH-shift and DOT-shift integrated fed-batch fermentation process for the production of ganoderic acid and *Ganoderma* polysaccharides by medicinal mushroom *Ganoderma lucidum*. *Bioresource Technology*, *100*, 1852–1859.

Wagner, R., Mitchell, D. A., Sassaki, G. L., & Amazonas, M. A. L. A. (2004). Links between morphology and physiology of *Ganoderma lucidum* in submerged culture for the production of exopolysaccharide. *Journal of Biotechnology*, *114*, 153–164.

Wang, Y., & McNeil. (1995). Production of the fungal exopolysaccharide scleroglucan by cultivation of *Sclerotium glucanicum* in an airlift reactor with an external loop. *Journal of Chemical Technology and Biotechnology*, *63*, 215–222.

Wasser, S. P. (2002). Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Applied Microbiology and Biotechnology*, *60*, 258–274.

Wasser, S. P. (2010). In P. M. Coates, J. M. Betz, M. R. Blackman, G. M. Cragg, M. Levine, J. Moss, & J. D. White (Eds.), *Reishi. Encyclopedia of dietary supplements*. Informa Healthcare.

Yang, F.-C., & Liau, C.-B. (1998). The influence of environmental conditions on polysaccharides formation by *Ganoderma lucidum* in submerged cultures. *Process Biochemistry*, *33*, 547–553.

Ye, L., Zhang, J. S., Zhou, K., Yang, Y., Zhou, S., Jia, W., et al. (2008). Purification, NMR study and immunostimulating property of a fucogalactan from the fruiting bodies of Ganoderma lucidum. *Planta Medica*, *74*, 1730–1734.

Ye, L. B., Zhang, J. S., Ye, X. J., Tang, Q. J., Liu, Y. F., Gong, C. Y., et al. (2008). Structural elucidation of the polysaccharide moiety of a glycopeptide (GLPCW-II) form *Ganoderma lucidum* fruiting bodies. *Carbohydrate Research*, *343*, 746–752.

Ye, L. B., Zhang, J. S., Yang, Y., Zhou, S., Liu, Y. F., Tang, K. J., et al. (2009). Structural characterization of a heteropolysaccharide by NMR spectra. *Food Chemistry*, *112*, 962–966.

Ye, L. B., Li, J. R., Zhang, J. S., & Pan, Y. J. (2010). NMR characterization for polysaccharide moiety of a glycopeptide. *Fitoterapia*, *81*, 93–96.