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Influence of Substrate Composition on β-Glucans Production and Growth of *Ganoderma lucidum*

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Abstract

Ganoderma lucidum is a medicinal mushroom known and used for centuries in China, claimed as beneficial for health due to the immunological effects provided by (1-3)β, (1-6)β-glucans present in its cell wall. Agricultural residues can be used as substrate for solid-state fermentation and turned into a product rich in β-glucans, that can be used for animal feeding, enhancing the immune response and, thus, reducing the utilization of antibiotics and other drugs. Therefore, colonization rate (growth), yield and concentration of (1-3)β, (1-6)β-glucans of different agricultural residues, such as soybean hulls, soybean residue and corn residue after solid state fermentation with *G. lucidum* were determined and evaluated according to their composition before fermentation. Specific growth rate (k) was higher for soybean hulls (k_1 = 0.165) and corn residue (k_3 = 0.161), but concentration of (1-3)β, (1-6)β-glucans was higher in soybean residue (234.09 mg g^{-1}) and soybean hulls (180.32 mg g^{-1}). Considering the nutritional composition of substrates, the concentration of (1-3)β, (1-6)β-glucan can be related to the ratio between fiber carbohydrates and total carbohydrates, demonstrating that fiber is an important feature regarding the production of β-glucans by the fungus. Also, colonization rate can be related to the total carbohydrates concentration and total carbohydrates/crude protein ratio, showing that carbohydrates and proteins have an important effect over the growth of the fungus. Soybean hulls showed to be the most feasible substrate for *G. lucidum* mycelia production presenting high concentration of (1-3)β, (1-6)β-glucans and colonization rate, with potential to be a dietary supplement for farm animals.

Keywords: fermentation, fiber, fungi

1. Introduction

Ganoderma lucidum, known as Reishi in Japan, and Lingzhi in China, is a basidiomycete, wood decaying mushroom, traditionally used in China for centuries and considered as a medicinal mushroom that enhances health and promotes longevity. Commercialization and manufacturing of this mushroom and its products represent an economic impact of 2.5 million dollars in the United States (Bishop et al., 2015). The commercial products can be found as fruiting body (as teas, powder or capsules), spores (capsules) and mycelium (in grains or capsules). However; it takes 45 to 150 days to produce fruiting bodies and spores, depending on genetics and environmental factors such as temperature, humidity and photoperiod (Rolim, Sales-Campos, Cavalcanti, & Urben, 2015).

Substances that provide therapeutic characteristics to this mushroom, named bioactive compounds, include polysaccharides, dietary fibers, oligosaccharides, triterpenoids, peptides, proteins, alcohols, phenols, mineral elements, vitamins and amino acids. The therapeutic properties are directly related to the immunological properties of those substances, and (1,3)β, (1,6)β-glucans are the most studied (Batra, Sharma, & Khajuria, 2013).

Mycelium of *G. lucidum* contains the same bioactive compounds of fruiting body at similar concentrations, and the time for production is very much lesser, varying from 7 to 30 days (Liu, Shen, Xia, Zhang, & Park, 2012). Several researches have been conducted aiming to improve its production in different culture media, both in

solid or liquid media. There are few researches about solid media cultivation or solid-state fermentation, due to the difficulty in separating the mycelium from the substrate (Elisashvili, 2012).

Still, cultivation of *G. lucidum* mycelium in solid medium is an alternative when all substrate can be used as feed, such as cereal grains fermentation, from which flour can be made and used in cooking recipes. The use of agricultural residues is an alternative that have been studied, aiming the incorporation of this product for animal feeding (Dinis et al., 2009; Graminha et al., 2008; Rodrigues et al., 2008; Shrivastava et al., 2012). The proposal of new types of food and dietary supplements for antibiotic replacement and antiviral agents for farm animals is one of the perspectives on medicinal mushroom research (Wasser, 2014).

Some researchers, already observed the ability of this mushroom to grow in soy residues, rice residues, cheese whey, deproteinated cheese whey, among others agricultural residues (Hsieh & Yang, 2004; Lee, Song, Yu, & Hwang, 2003a; Lee, Song, & Hwang, 2003b; Yang, Hsieh, & Chen, 2003; Shi, Zhang, & Yang, 2013). The use of residues in mushroom production represents a valuable conversion of low nutritive phytomass into enriched food and also an alternative to minimize environmental impact that may be caused by the incorrect discard (Smil, 1999, Van Zanten et al., 2014).

Therefore, growth of *G. lucidum*, as well as its yield and concentration of β-glucans in soybean hulls, soybean residue and corn residue were analysed considering their nutritional composition, in order to determine which would be the most feasible to be used as supplement in animal feeding.

2. Materials and Methods

Ganoderma lucidum CC339ST was obtained from Brazilian Agricultural Research Corporation – Genetic Resources and Biotechnology (EMBRAPA Cenargen). Cultures were inoculated on Potato Dextrose Agar (PDA) petri dishes and incubated at 28 °C for 7 days, and stored for 3 months at 4 °C until the beginning of the experiment. Mycelium was activated on PDA with the same procedure and then used for the inoculation in the residues, immediately after 7 days of incubation. This activation considers that the metabolism of the fungus probably slows down as a result of the storage at low temperatures, then 'activation' is needed for an adequate metabolic rate, assuring uniform growth.

The agricultural residues were soybean hulls, soy residue and corn residue. "Soybean hulls" is an agricultural by-product obtained from the production of soybean oil commonly used in animal feeding. Soy residue and corn residue were obtained during the loading of grains from the trucks to the silos through a rolling mat. During this process, minor particles such as hulls, broken grains and germens were 'sieved' through the mat resulting in a residue with no commercial value.

Residues were macroscopically analysed for the presence of strange particles (little rocks, insects, etc.), then washed and sieved in a 250 μm sieve. All of them were processed according to Urben (2004) for "Seed production" with the following adaptations. The residues were boiled for 2 minutes and then pressed against a 250 μm sieve until no water could be drained. Each residue was packed in polypropylene bags and weighted at 200 g and added with 1% of $CaCO_3$ (w/w). All bags were closed with cotton plugs and sterilized at 121 °C for 20 minutes. After sterilization, 0.25 cm^2 of mycelial agar were placed asseptically on residues and incubated at 28 °C for the growth measurements.

Mycelial growth was measured using self-designed frame that contained the propylene bag allowing measurements in four sections of the periphery with a ruler. The measures in the four sections were made at a 3 day interval from day 0 until complete colonization of the substrate that was achieved when no growth could be measured. After complete colonization, samples were dehydrated in Pasteur oven at 55 °C until constant weight and freezed at -4 °C until laboratorial analysis (Figure 1).

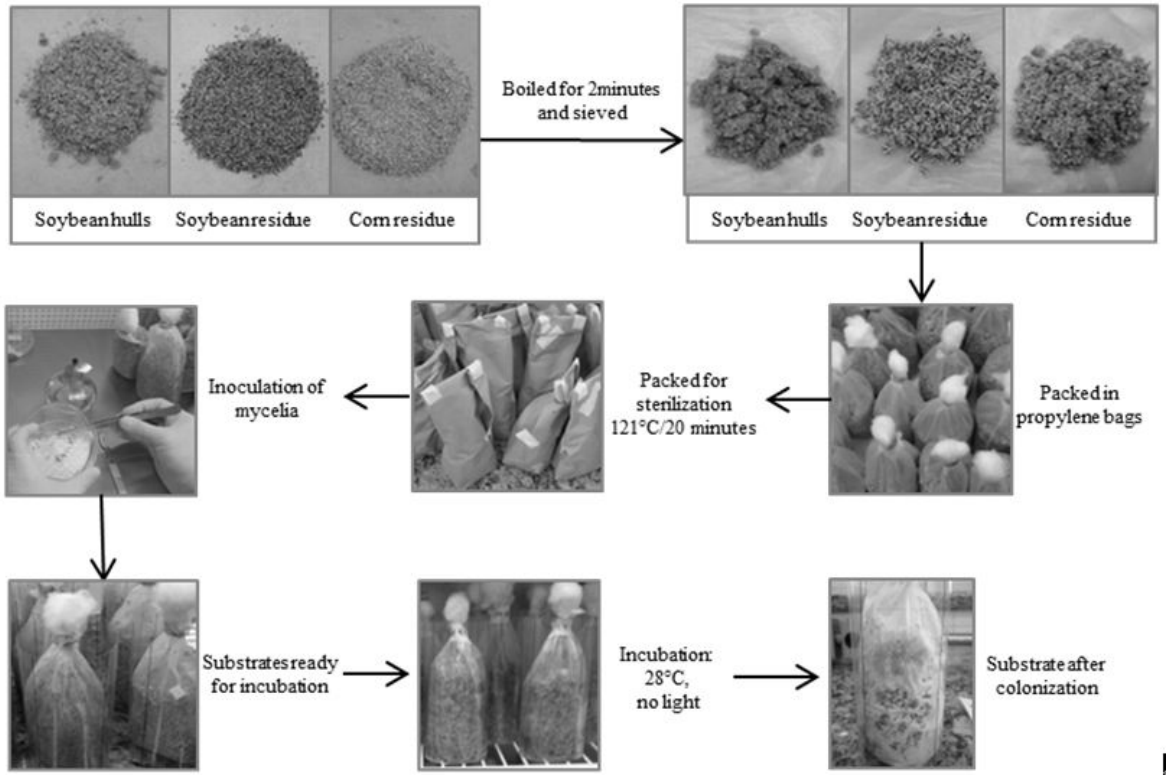

Figure 1. Methodology for inoculation of mycelium and incubation for colonization by *Ganoderma lucidum*

Yield was calculated using the following equation:

$$Yield\ (\%) = (Final\ weight/Initial\ weight) \times 100 \quad (1)$$

Nutritional composition (Table 1) was performed with the following analytical process according to the Association of Official Analytical Chemists [AOAC] (1995), dry matter, ashes, crude protein and fat; for neutral detergent fiber according to Mertens (2002). After colonization, samples were analysed for (1-3)β, (1-6)β-glucans according to Lever (1972) using β-glucanase enzyme (Sigma®) and reaction with hydrazide.

Table 1. Nutritional composition (means and standard deviation) of agricultural residues used in solid fermentation with *G. lucidum*

	Soybean hulls	Soybean residue	Corn residue
DM (%)	86.81 ± 0.02	86.38 ± 0.35	86.54 ± 0.75
Ash (%)	3.92 ± 0.18	11.92 ± 0.16	0.85 ± 0.02
OM (%)	96.08 ± 0.18	88.08 ± 0.16	99.15 ± 0.08
CP (%)	10.86 ± 0.68	40.36 ± 0.40	11.03 ± 0.52
Fat (%)	0.85 ± 0.01	3.11 ± 0.06	1.38 ± 0.06
NDF (%)	61.02 ± 0.33	22.49 ± 0.17	33.83 ± 1.30
TC	84.37	44.61	86.74
NFC	23.35	22.12	52.91
TC/CP	7.77	1.11	7.86
NFC/TC	0.28	0.50	0.61
NDF/TC	0.72	0.50	0.39

Note. DM: Dry Matter; OM: Organic Matter; CP: Crude Protein; NDF: Neutral Detergent Fiber; TC: Total Carbohydrates; NFC: Non-Fiber Carbohydrates. TC and NFC were calculated according to Sniffen, O'Connor, Van Soest, Fox and Russel (1992): TC = 100 – (CP + Fat + Ash); NFC = TC – NDF.

The trial was performed in a completely randomized design with 3 treatments and 9 replicates. Data of yield and β-glucans were analysed with Bartlett and Shapiro-Wilk to test the variance homogeneity and the normal distribution, respectively. Data was transformed by Box Cox method when distribution was not normal with packages AID and car of RStudio® (v. 3.2.1; The R Foundation for Statistical Computing). After checking the normality and the variance homogeneity the ANOVA test was performed and when the nullity hypothesis (H_0) was rejected the treatment means of these variables were compared by Tukey test. All the statistical tests were computed by using the packages ExpDes.pt and ggplot2 of the RStudio® (v. 3.2.1; The R Foundation for Statistical Computing) considering $\alpha = 0.05$.

The average of the measures taken at the four sections (cm) was used to estimate mycelial growth. This data was adjusted using the following equation:

$$Y = A - B \exp(-kt) \quad (2)$$

This equation represents the monomolecular growth model (Brody, 1945; France, Dijkstra, & Dhanoa, 1996), in which A is the asymptotic size (cm), k is the first-order specific growth rate (day^{-1}) and B is a scale parameter. The parameter estimation was performed by using the NLMIXED procedure of SAS (v.9.4, SAS Systems, Inc., Cary, NC, USA). Attempts were made to correct for heterogeneity of variances over time in nonlinear parameter estimation (Matis & Hartley, 1971; Bard, 1974) by using three types of special parametric structure on the variance and covariance matrix (Littell, Milliken, Stroup, Wolfinger, & Schabenberger, 2006): Variance Component (VC), that specifies standard variance components, Unstructured (UN) that specifies a completely general unstructured covariance matrix parameterized directly in terms of variances and covariances, and Spatial Power (SP(POW)) that specifies the spatial power structures, used when the correlation declines in function of time. This type of methodology has been computationally feasible only in recent years (Pinheiro & Bates, 2000; Littell et al., 2006; Vonesh, 2012). The choice of the better matrix to represent the variance structure was accomplished by computing Akaike's Information Criterion - AIC_{cr} (Akaike, 1974; Burnham & Anderson, 2004), considering that the best structure would be the one that presented the lowest AIC_{cr} value.

3. Results and Discussion

Yield (the amount of resulting product after mycelial growth) was below 30% in all residues (Table 2) and the residue that presented lower yield was soybean hulls (15.01%). Hsieh and Yang (2004) used soy residue from the waste of tofu manufacturing and Yang et al. (2003) used stillage grain from a rice-spirit distillery and did not determined yield, because they considered the appearance of fruiting bodies as result. Other researchers that used solid substrates considered the harvest of fruiting bodies (g) as a ratio of the substrate (kg) to calculate the yield, such as, Peksen and Yakupoghu (2009) that used tea waste and observed the higher yield of 87.98 g of mushroom kg of $substrate^{-1}$; and Erkel (2009) that used different types of sawdust and observed 68.44 g of mushroom kg of $substrate^{-1}$ as higher yield.

This work considered the substrate with the mycelium as final product, because of the possibility to include it in animal feeding. According to Graminha et al. (2008), solid state fermentation represents a potential for animal feeding in developing countries because it enhances nutritive value of agricultural residues without the need of high technology. Aiming animal feeding, Rodrigues et al. (2008) evaluated digestibility and Dinis et al. (2009) evaluated the lignin modification of wheat straw fermented with different white-rot fungi. While Shrivastava et al. (2012) evaluated wheat straw fermented with *Ganoderma* sp. rckk02 and observed an enhance in nutritional value as well as digestibility.

Table 2. Yield (%), (1-3) β, (1-6) β-glucan (mg/g) and protein (mg/g) of agricultural residues for solid state fermentation with *G. lucidum* (means and standard deviation)

Trait	Soybean hulls	Soybean residue	Corn residue
Yield	$15.01^{c} \pm 2.76$	$23.38^{b} \pm 1.69$	$27.86^{a} \pm 4.78$
(1-3)β, (1-6)β-glucan	$180.32^{a} \pm 34.33$	$234.09^{a} \pm 49.44$	$6.53^{b} \pm 1.57$
Protein	$1.54^{b} \pm 0.46$	$2.27^{ab} \pm 0.35$	$2.70^{a} \pm 1.13$

Note. Values followed by different letters among residues are significantly different by Tukey test $\alpha = 0.05$.

Concentration of (1-3)β, (1-6)β-glucan data was not normally distributed and, therefore, was transformed by Box Cox for analysis with Tukey test, resulting in higher concentration of β-glucans (Table 2) for soybean residue (234.09 mg g^{-1}) and soybean hulls (180.32 mg g^{-1}). The use of a solid substrate requires a specific enzime for

determining (1-3)β, (1-6)β-glucan, because other glucans, such as (1,3)β, (1-4)β-glucan can be found in the cell wall of plants and in cereal grains (Hall, 2003).

Data for β-glucans, could only be found from experiments that used submerged cultivation, and have been referred as exopolyssacharides (EPS). In those cases, the liquid culture media was filtered and the remaining biomass from *Ganoderma* was added with NaOH or ethanol for EPS precipitation, then the sample could be analysed by phenol-sulfuric acid method (Wagner et al., 2003). Yuan, Chi, and Zhang (2012) used several glucose concentrations, sources of potassium and magnesium and different C/N ratios in liquid media for Ganoderma cultivation in shaking flasks and reached an EPS production of 1 723 mg L^{-1}. Wagner et al. (2004) obtained EPS of 5 700 mg L^{-1} using culture media added with glucose at pre-established intervals.

Considering the nutritional composition of the residues, a positive relationship can be observed between the concentration of (1,3)β, (1,6)β-glucans and the ratio between fibrous carbohydrates and total carbohydrates (NDF/TC) (Table 1). This positive relationship is possible because basidiomycetes are fungi that degrade cell wall of plants and *G. lucidum* is the richest in carbohydrate-active enzymes (CAZymes), with an apparatus of 417 genes related to these enzymes. Its genome codifies enzymes for the three main classes of polyssacharides of the cell wall of plants: cellulose, hemicellulose and pectin. *G. lucidum* is also the one that gather the wider and complete collection of lignolytic peroxydases together with laccases and one cellobiose dehydrogenase (Chen et al., 2012).

Asymptotic size (parameter A) and specific growth rate (parameter k) were studied through adjustment to the equation of monomolecular growth model (Brody, 1945; France et al., 1996) and analysed for the best parametric structure. This study resulted in the UN matrix (AIC_{cr} = 510.4) as the best parametric structure for the variance and covariance heterogeneity, when compared to VC matrix (AIC_{cr} = 869.8) and SP(POW) matrix (AIC_{cr} = 589.1). Ganoderma mycelia developed very well in all residues, despite the difference of time for complete colonization (21 days for corn residue and 39 for others). Growth happened in a descendent way, but in soybean hulls descendent growth ceased approximately after 30 days, probably due to the humidity of the substrate, and began an ascendant growth towards the cotton plug of the bag. This behavior can be explained by the changes in pressure, volume and flux of water that enters and leaves the cell. These changes interfere directly in cell turgor and in the ability of the hyphae tip for growth Thus, when the humidity of substrate was higher, hyphae began to grow towards the most favorable environment. This behavior can be depicted through the results from the equation with similar asymptotic size (Table 3, Figures 2 and 3) for soybean residue (A2 = 12.417) and corn residue (A3 = 12.142), but lower for soybean hulls (A1 = 7.168).

Table 3. Point and interval estimates of the parameters of the Brody model of growth

Parameter	Estimate	Standard Error	Asymptotic confidence interval at the probability level of 0.95	
			Lower	Upper
A1	7.168	0.240	6.671	7.665
A2	12.417	0.238	11.926	12.908
A3	12.142	0.240	11.646	12.638
B1	16.257	0.772	14.662	17.853
B2	18.058	0.547	16.927	19.188
B3	23.355	0.754	21.797	24.913
k1	0.165	0.008	0.148	0.182
k2	0.092	0.003	0.086	0.099
k3	0.161	0.005	0.150	0.172

Note. A = Asymptotic size; B = Scale parameter; k = Specific growth rate; 1 = Soybean hulls; 2 = Soybean residue; 3 = Corn residue.

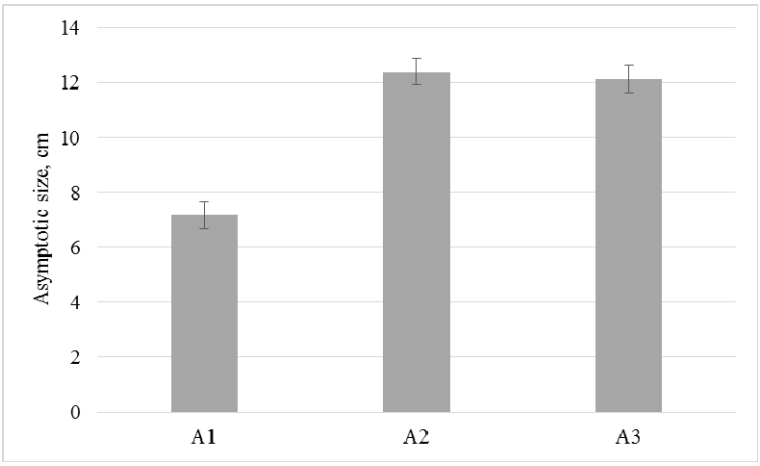

Figure 2. Point and confidence interval for parameter A (asymptotic size)

Note. A1 = Soybean hulls; A2 = Soybean residue; A3 = Corn residue.

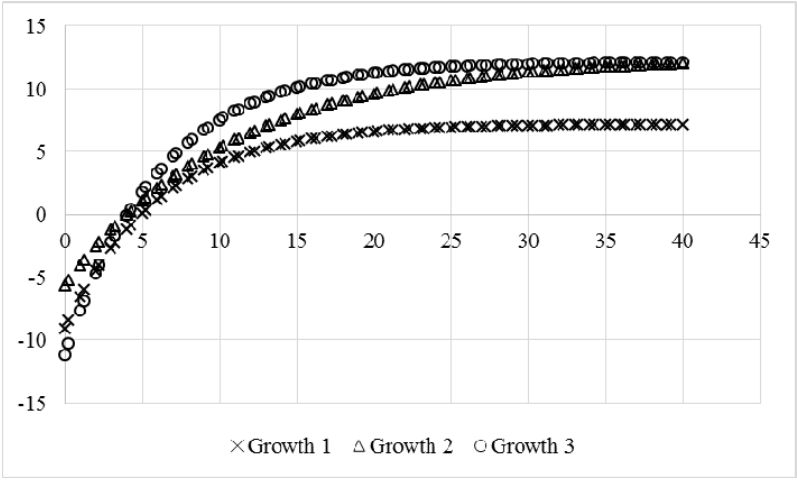

Figure 3. Linear growth behavior of *Ganoderma lucidum* as a function of time on the different residues

Note. Growth 1 = Soybean hulls; Growth 2 = Soybean residue; Growth 3 = Corn residue.

Despite the results for asymptotic size, the especific growth rate (Table 3, Figures 3 and 4) of soybean residue was low (k2 = 0.092), whereas soybean hulls (k1 = 0.165) and corn residue (k3 = 0.161) were similar. These results mean that soybean residue needed more time than soybean hulls and corn residue to achieve the asymptotic size. Considering the nutritional composition (Table 1) the residues that presented higher and similar colonization rate also showed higher and similar total carbohydrates (TC) and total carbohydrates/crude protein ratio (TC/CP).

Hsieh and Yang (2004) and Yang et al. (2003) measured growth linearly in $mm.day^{-1}$, but a mathematical model should be used because the fungus presents a biological pattern of growth. Tang and Zhong (2004) used a kinetic model (Monod model) to describe the cell growth (dry cell weight) of Ganoderma in submerged cultivation and found an estimated specific growth rate of 0.23.

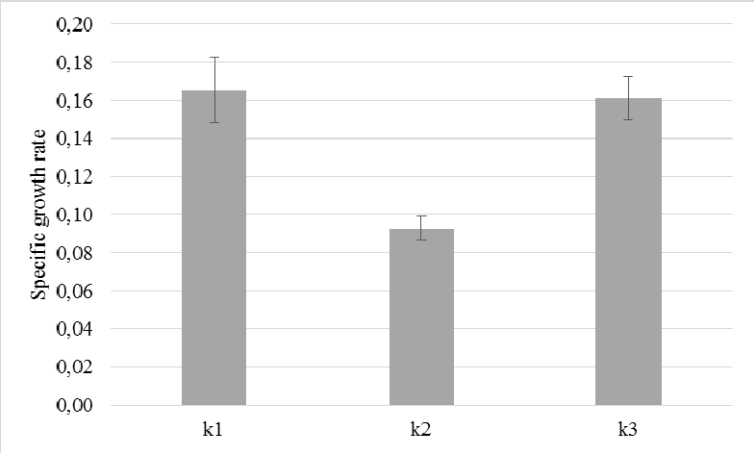

Figure 4. Point and confidence interval for parameter k (specific growth rate)

Note. k1 = Soybean hulls; k2 = Soybean residue; k3 = Corn residue.

Carbohydrates are important for the growth of fungi, because the cell wall synthesis depends on glucose supply. During the cell wall synthesis, glucose is transported by uridine diphospho glucose (UDPGlc) using the GTP activating enzyme (1-3)β-glucan synthase for (1-3)β-glucan synthesis, an important component of the cell wall (Bartnicki-Garcia, Bracker, Gierz, López-Franco, & Lu, 2000; Wessels, 1994). Proteins are essential for synthesis of enzymes, such as those related to substrate degradation and those related to cell growth. All these enzymes are found in the tip of the hyphae that presents all physical requirements for cell growth: plastic deformation, incorporation of new wall and membrane material (Lew, 2011). Therefore, TC and TC/CP were determinative for the colonization rate of the substrate, supplying glucose and specific enzymes for cell growth of the fungus. However, the excess of CP can impair the specific growth rate as can be observed from the results of soybean residue.

4. Conclusions

Soybean hulls enriched with *G. lucidum* mycelium may be feasible to be used as dietary supplement for farming animals because presented the adequate nutritional composition resulting in high concentration of (1-3)β, (1-6)β-glucan and high colonization rate when compared to soybean residue and corn residue.

The results of this work demonstrate that the presence of fibrous carbohydrates influences the concentration of (1-3)β, (1-6)β-glucan, whereas growth is influenced by total carbohydrates and the ratio between total carbohydrates and proteins. Further researches must consider the fibrous carbohydrates as an important component of culture medium or substrate for production of (1-3)β, (1-6)β-glucan with *Ganoderma lucidum*.

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Appendix

Appendix A. R codes used in this paper:

```
> setwd("~/RSTUDIO")
> data <-read.csv2("~/RSTUDIO/data.csv")
> attach(data)
> require (ExpDes.pt)
> require (ggplot2)
> require (AID)
> require (car)
> bartlett.test(variable~treatment, data)
> m=lm(variable~factor(treat), data)
> shapiro.test(resid(m))
> boxcoxnc (data, method = “sw”, lam = seq (-3,3,0.01), plotit = TRUE, alpha verbose = TRUE)
> dic(treat,variable,quali=TRUE,mcomp="tukey",sigT = 0.05,sigF = 0.05)
```