MYCELIAL BIOMASS CULTIVATION OF *Lentinus crinitus*

CRESCIMENTO MICELIAL DE Lentinus crinitus

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ABSTRACT: *Lentinus crinitus* is a medicinal basidiomycete, little studied regarding the basic cultivation conditions, which is used in bioremediation and consumed by native Indians from the Brazilian Amazon. Also, it produces a fungal secondary metabolite panepoxydone that has been described as an essential regulator of the inflammatory and immune response. This study aimed to evaluate basic conditions of temperature, pH, and nitrogen concentration and source in the cultivation of *L. crinitus* mycelial biomass. In order to evaluate fungal growth temperature, 2% malt extract agar (MEA) medium, pH 5.5, was utilized from 19 to 40 °C. For pH, MEA had pH adjusted from 2 to 11 and cultivated at 28 °C. Urea or soybean meal was added to MEA to obtain final concentration from 0.5 and 16 g/L of nitrogen, pH of 5.5, cultivated at 28 °C. The best temperature growth varies from 31 to 34 ºC and the optimal one is 32.7º C, and the best pH ranges from 4.5 to 6.5 and the optimal one is 6.1. Protein or non-protein nitrogen concentration is inversely proportional to the mycelial biomass growth. Nitrogen concentrations of 2.0 g/L soybean meal and urea inhibit mycelial biomass growth in 11% and 12%, respectively, but high concentrations of 16.0 g/L nitrogen inhibit the growth in 46% and 95%, respectively. The fungus is robust and grows under extreme conditions of temperature and pH, but smaller adaptation with increasing nitrogen concentrations in the cultivation medium, mainly non-protein nitrogen.

KEYWORDS: Axenic cultivation. Cultivation condition. Mycelial biomass growth. Soybean. Urea.

INTRODUCTION

Lentinus crinitus (L.) Fr. is a wild basidiomycete, native to Brazil, with pantropical (tropical regions of Africa, Asia and America) and neotropical (Central America, southern Florida, Caribbean islands and South America) distribution, frequently found in decomposing tree trunks (SILVA; GIBERTONI, 2006). This fungus grows in several substrates and is utilized in bioremediation (BALLAMINUT; MATHEUS, 2007), product discoloring in the textile industry (SANCHEZ-LOPEZ et al., 2008; NIEBISCH et al., 2010; ALMEIDA et al., 2018; MARIM et al., 2018) and presents a powerful arsenal of enzymes dedicated to the breakdown and consumption of lignocellulose (VALLE et al., 2014; CAMBRI et al., 2016; CONCEIÇÃO et al., 2017; SANTANA et al., 2018). Moreover, it produces a fungal secondary metabolite panepoxydone that has been described as an inhibitor of NF-κB activation, and it is a regulator of the inflammatory and immune response (ERKEL; WISSER; ANKE, 2007; WASSER, 2010; CHANG; WASSER, 2012; WASSER, 2017). However, few studies are related to the specific conditions of *L. crinitus* mycelial biomass growth and usually the ones that are utilized are established for other basidiomycetes such as *Agaricus brasiliensis* (COLAUTO et al., 2008), *Lentinula edodes* (FENG et al., 2010), *Lentinus sajor-caju* and *Pleutorus* spp (ALBUQUERQUE, NOGUEIRA; NASCIMENTO, 2012).

Mycelial biomass production is an alternative to mushroom production due to a better utilization and standardization of the substrate and better contaminant control (EIRA, 2004), mainly for the production of bioactive molecules. *L. crinitus* is particularly interesting for the mycelial biomass production since this mushroom consumption is little appreciated due to its fibrous texture, despite being consumed by the Sanama (*Yanomami*) Indians (FIDALGO; PRANCE, 1976; VARGAS-ISLA; ISHIKAWA, 2008). Moreover, this fungus produces panepoxydone, a bioactive fungal metabolite, which can be effectively used against malignant cells, mainly breast cancer (ERKEL; ANKE; STERNER, 1996). Unlike synthetic compounds, fungal metabolites can penetrate into target cell membrane easily, increase its bioefficacy due to the low molecular size, and improve the overall human immune system (SHARMA; ANNEPU, 2018).

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However, for the mycelial biomass production, it is important to determine the optimal cultivation and variation conditions in which the fungus can grow such as the temperature range, initial pH, and nitrogen concentration and source in the cultivation medium (COLAUTO et al., 2008). Temperature is one of the most important factors that affect the mycelial biomass growth among mushroom-producing basidiomycetes. It has such an important role in the fungus adaptation regarding the enzymatic activity that it causes alterations in the metabolism and mycelial biomass growth (MILES; CHANG, 1997). Another important variable that affects the mycelial biomass growth is the pH. Fungi are usually limited to grow in a narrow range of pH, close to neutrality, although some may tolerate extreme pH; this parameter can directly affect the cell membrane and the level of ion dissociation, interfering in the absorption of nutrients and minerals of the environment (DEACON, 2006). The adaptation to different pHs requires a homeostatistic system of internal pH and a specific regulatory system which guarantees that the molecules exposed to the environment be secreted only under favorable conditions (PEÑALVA et al., 2008). Another requirement is the nitrogen concentration and source that directly affects the metabolization capacity of the fungus for the mycelial biomass growth and colonization of different types of substrates under different ambient conditions, and ammonium and glutamine sources were the preferred ones (MARZLUF, 1997). In the absence of nitrogen sources, other sources may be utilized, although they are less easily assimilated such as nitrate, urea, uric acid, amines, amides, purines, and pyrimidines (MARZLUF, 1997; WONG; HYNES; DAVIS, 2008). D'Agostini et al. (2011) evaluated the effect of the addition of non-protein sources and concentrations on the mycelial biomass growth of *Pleurotus ostreatus*, *L. edodes* and *A. brasiliensis* and concluded that ammonium sulfate, ammonium:urea sulfate (1:1), and urea are more appropriate. Das et al. (2015) evaluated *Lentinus squarrosulus* in submerse cultivation through several nitrogen sources, with greater mycelial biomass growth with yeast extract, a protein nitrogen source, and a smaller growth with ammonium nitrate, a non-protein nitrogen source. Due to the lack of basic and specific parameters for the axenic cultivation of *L. crinitus*, this study aimed to evaluate the temperature, initial pH, and nitrogen concentration and source in the cultivation medium for the mycelial biomass growth of *L. crinitus* in order to develop the axenic cultivation of this fungus.

MATERIALS AND METHODS

Biological material

L. crinitus U9-1 strain from the culture collection of the Laboratory of Molecular Biology of Paranaense University was previously identified by sequencing the internal transcribed spacers (ITS) of ribosomal DNA (MARIM et al., 2018) and deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/), accession number MG211674. The cryopreserved fungus (MANTOVANI et al., 2012) was recovered on malt extract (20 g/L; Himedia®) and agar (5 g/L; Himedia®), previously autoclaved at 121 °C for 20 min (TANAKA et al., 2019). The fungus was kept at 25 ± 1 °C, in the dark for seven days. The inoculum was selected from the mycelial growth edge with uniform appearance and without sectioning.

In addition, incorrect names, synonyms, and eventual typing mistakes or misspellings can lead to confusion and, therefore, the current name should be clear. *L. crinitus* (current name) has 25 synonyms such as *Agaricus crinitus* L., *Agaricus essequeboensis* G. Mey., *Lentinus chaetoloma* Fr., *Lentinus crinitus* var. *crinitus* (L.) Fr., *Lentinus crinitus* var. *denudatus* Pilát, *Lentinus crinitus* var. *squamulosus* Pilát, *Lentinus crinitus* var. *subcervinus* (Berk. & M. A. Curtis) Pilát, *Lentinus crinitus* var. *subcrinitus* Pilát, *Lentinus crinitus* var. *subvillosus* Pilát, *Lentinus crinitus* var. *typicus* Pilát, *Lentinus essequeboensis* (G. Mey.) Fr., *Lentinus microloma* Pat. & R. Heim, *Lentinus rigidulus* Berk. & M. A. Curtis, *Lentinus subcervinus* Berk. & M. A. Curtis, *Lentinus wrightii* Berk. & M. A. Curtis, *Panus crinitus* (L.) Singer, *Panus crinitus* var. *crinitus* (L.) Singer, *Panus wrightii* Berk. & M. A. Curtis, *Pocillaria chaetoloma* (Fr.) Kuntze, *Pocillaria crinita* (L.) Kuntze, *Pocillaria essequeboensis* (G. Mey.) Kuntze, *Pocillaria rigidula* (Berk. & M. A. Curtis) Kuntze, *Pocillaria subcervina* (Berk. & M. A. Curtis) Kuntze, *Pocillaria wrightii* (Berk. & M. A. Curtis) Kuntze, and *Polyporus phyllostipes* D. Krüger (KIRK, 2019).

Mycelial biomass growth under different cultivation conditions

In order to evaluate the effect of temperature, the fungus was grown in 2% malt extract agar (MEA) (20 g/L malt extract and 10 g/L agar; Himedia®), with initial pH of 5.5, and cultivated from 19 to 40 °C, with variation every 3 °C. For the evaluation of the effect of pH, the fungus was grown in MEA at 28 °C with pH

adjusted from 2 to 11, varying every 0.5 units, adjusted with HCl (1 mol/L) or NaOH (1 mol/L), previously filtered (⌀ 0,22 µm). In order to assess the effect of nitrogen concentration and source, non-protein nitrogen (urea; CH_4N_2O) or protein nitrogen (soybean meal) was added to MEA to obtain final concentration of 0,5, 1, 2, 4, 6, 8, 10, 12, 14, and 16 g/L of nitrogen in the cultivation medium, with pH of 5.5 and cultivation at 28 °C. An MEA disk of 0.5 mm of diameter, containing mycelia, was inoculated in the middle of the cultivation medium for mycelial grown for 28 days in the dark, and the diameter of the mycelium radial growth was measured from four equidistant points to obtain arithmetic average.

Statistical analysis

All the treatments were performed randomly and were replicated four times each. The data were evaluated by analysis of variance and the significant differences were determined by Tukey test ($p \leq 0.05$). The ideal values of mycelial biomass growth were determined by regression.

RESULTS

The fungus was able to grow from 19 to 40 ºC, but the temperature range that promotes the greatest ($p \leq 0.05$) mycelial biomass growth was from 31 to 34 ºC (Fig. 1). By polynomial regression ($R^2 = 0.97$), the optimal temperature peak of the mycelial biomass growth was 32.7 ºC (Figure 1).

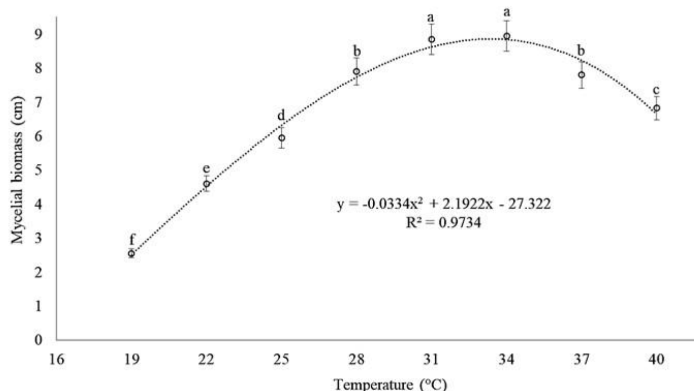

Figure 1. Diameter of *Lentinus crinitus* mycelial biomass growth in 2% malt extract agar, initial pH of 5.5, and cultivated at different temperatures.
Different letters indicate differences ($p \leq 0.05$) by Tukey test (n = 4).

The fungus was capable of growing in a cultivation medium with the initial pH from 2.5 to 10.5, but there was no mycelial biomass growth with pH from 2 or 11 (Figure 2). The greatest ($p \leq 0.05$) values of mycelial biomass growth were with pH from 4.5 and 6.5 and by polynomial regression ($R^2 = 0.95$) the optimal pH peak for mycelial biomass growth was 6.1 (Fig. 2).

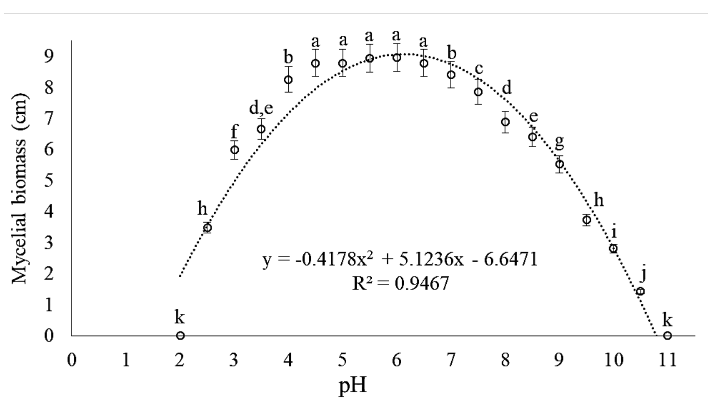

Figure 2. Diameter of *Lentinus crinitus* mycelial biomass growth in 2% malt extract agar and cultivated at 28 ± 1 ºC with different values of initial pH in the cultivation medium.
Different letters indicate differences ($p \leq 0.05$) by Tukey test (n = 4).

The greatest nitrogen concentration in the cultivation medium inhibited ($p \leq 0.05$) the mycelial biomass growth, regardless of the nitrogen source (Figure 3). The addition of 2.0 g/L of non-protein source inhibited the mycelial biomass growth in 11.9%, while the addition of 16.0 g/L inhibited in 95.1% (Fig. 3), indicating a low metabolization capacity of urea. The addition of protein nitrogen also inhibited the mycelial biomass growth, but in a less intensive manner (Fig. 3). The addition of 2.0 g/L of protein nitrogen inhibited the mycelial biomass growth in 11.3%, while the addition of 16.0 g/L inhibited 45.6%, with a tendency of fungal adaptation without great effects on the mycelial biomass growth (Fig. 3) and indicating that this wild fungus prefers to grow under conditions with lower nitrogen concentration. There were no differences in the visual density of mycelial biomass ramification among the treatments.

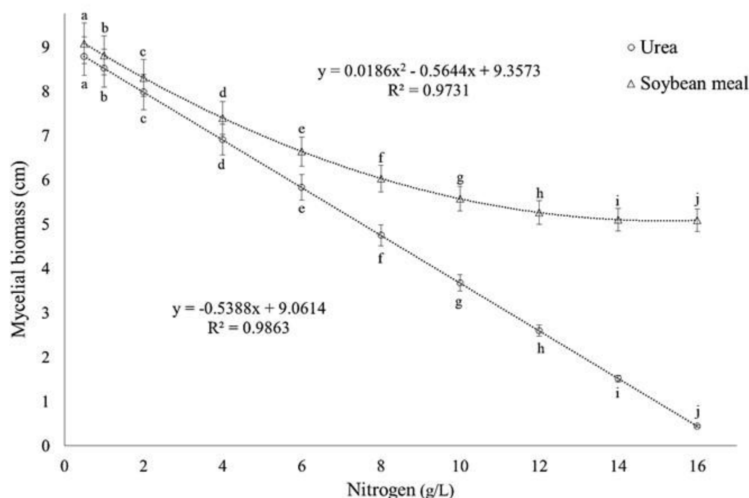

Figure 3. Diameter of *Lentinus crinitus* mycelial biomass growth in 2% malt extract agar with addition of protein (soybean meal) and non-protein (urea) nitrogen, initial pH of 5.5, and cultivated at 28 ± 1 ºC. Different letters in each linear or non-linear equation indicate differences ($p \leq 0.05$) by Tukey test (n = 4).

DISCUSSION

The optimal temperature peak for *L. crinitus* mycelial growth reported in our study was 32.7 ºC, and it is the first result of ideal temperature for mycelial biomass growth for this basidiomycete. The mycelial biomass grew from 19 to 40 ºC, with optimal range from 31 to 34 ºC. This ideal temperature range is higher than those reported for other edible commercialized basidiomycetes, which is 28 ºC for *A. brasiliensis* (COLAUTO et al., 2008), 30 °C for *Volvariella volvacea* (AKINYELE; ADETUYI, 2005), and 25 °C for *P. ostreatus* (SALES-CAMPOS et al., 2008) to name a few. However, Vargas-Isla and Ishikawa (2008) reported that the best temperature for mycelial biomass growth was at 35 °C to *Lentinus strigosus*, close to the value found in our study with *L. crinitus*. For Chang and Miles (2004), basidiomycetes can be classified into three groups according to the growth temperature: psychrophiles with a minimum temperature below 0 ºC, an optimum one around 17 ºC, and lack of growth above 20 ºC; mesophiles with minimum temperature above 0 ºC, maximum one below 50 ºC, and optimum temperature between 15 and 40 ºC; thermophiles with minimum temperature above 20 ºC, maximum one at or greater than 50 ºC, and optimum temperature around 35 ºC or higher. According to this classification, *L. crinitus* is in the highest temperature range in the group of mesophiles, close to thermophiles.

The temperature of mycelial biomass growth directly affects the energy expenditure and the production cost (COLAUTO et al., 2008). *L. crinitus* presents great growth from 31 to 34 ºC, but the growth at 28 or 37 ºC was still suitable. Thus, the adoption of optimal temperature for the mycelial biomass production will depend on the cost-benefit of the process and the possibility of energy reutilization in the process. In our study, this tendency of increase in the mycelial biomass up to 34 ºC indicates increase in the physiological reactions of the microbial growth. Fungi of *Lentinus* genus grow in tropical environments at high temperatures. Moreover, according to Mswaka and Magan (1999), a temperature of 35 °C was usually registered at midday inside tree logs. Thus, the *in vitro* growth results of our study are close to the ones found naturally in the growth environment of this fungus.

The maximum mycelial biomass growth was when the cultivation medium pH was 6.1. In general, an extreme pH (too basic or acid) denatures enzymes, making the mycelial biomass growth difficult. The optimal pH value obtained in our study for *L. crinitus* is close to the reported values of 5.5-6.0 for *A. brasiliensis* (COLAUTO et al., 2008), 4.5 for *P. ostreatus* (DIAZ et al. 2013), 6.5 for *V. volvacea* (SALES-CAMPOS et al., 2008), and 5-7 for *L. strigosus* (VARGAS-ISLA; ISHIKAWA, 2008).

The temperature and the pH of the cultivation medium mainly affect the speed of enzymatic reactions. Marim et al. (2018) evaluated the effect of temperature and initial pH in the liquid cultivation medium in the laccase activity of *L. crinitus* and reported a four-fold increase in the laccase activity when the optimal temperature was 28-30 °C and pH was 5.6-7.0. Therefore, the reported values of optimal temperature and pH for laccase production by *L. crinitus* are close to the ones obtained in our study of mycelial biomass growth. Marim et al. (2018) pointed out the differences of mycelial biomass growth and laccase activity for different strains of *L. crinitus* indicating the necessity of studies on bioproduct production for each strain.

In our study, an increasing addition of nitrogen in the cultivation medium inhibited the mycelial biomass growth of *L. crinitus.* The most significant inhibition occurred with non-protein nitrogen whereas the protein nitrogen was metabolically administered by fungus even in greater amounts. Niamke and Wang (2004) reported that the typical nitrogen sources are ammonium, nitrates and urea and in complex cultivation media are peptone, tryptone, yeast extract, wheat bran and digested protein. Chang and Miles (2004) cited that for basidiomycetes the nutrients must be readily accessible because most of the species have limited access to non-protein nitrogen such as nitrate and, therefore, the supply of a protein nitrogen source is essential. Bertéli et al. (2016) reported that the utilization of isolated soybean protein (protein nitrogen source) was more effective in the mycelial biomass growth of *A. brasiliensis*, without growth inhibition as occurred with sodium nitrate (non-protein nitrogen source). According to Robbins (1939) reported that all fungus species utilize organic nitrogen (protein nitrogen) and a few ones use inorganic nitrogen (non-protein nitrogen). Moreover, Chang and Miles (2004) reported that some nitrogen sources are more appropriate to mycelial biomass growth of basidiomycetes than others, and that the necessary quantity for fructification is slightly greater than the quantity that supports the mycelial biomass growth. Very high nitrogen concentrations can inhibit fructification and/or mycelial biomass growth by the accumulation of toxic metabolic products or depletion of essential metabolites. In addition, Zhang et al. (2007) reported that high nitrogen concentrations inhibit enzyme synthesis in basidiomycetes. Thus, studies with variation of nitrogen sources and concentration are needed to determine the mycelial biomass growth of each fungus.

Nevertheless, in our study, *L. crinitus* was collected from a decaying tree trunk in nature. D'Agostini et al. (2011) reported that *L. edodes* grows better in substrates with reduced nitrogen concentration. These fungi are likely adapted to substrates with high carbon/nitrogen ratio such as decaying tree logs with carbon/nitrogen ratio of approximately 500 (REIS et al., 2012), where they are more competitive compared to other microorganisms, providing a natural selective advantage.

Non-protein nitrogen such as urea or ammonium sulfate can be converted into ammonia (NH_3) which is toxic for the mycelium, and results in reduced degradation of lignin in the cultivation of *Pleurotus* spp (RAJARATHNAM, BANO; STEINKRAUS, 2009). In our study, the nitrogen concentration (urea) inhibited inversely proportionally to the mycelial biomass growth of *L. crinitus* until complete inhibition. Silva et al. (2007) reported that the nitrogen concentration of 20.0 g/L in the cultivation medium completely inhibited the mycelial biomass growth of *Pleurotus sajor-caju*, while in our study the concentration of 16.0 g/L inhibited 95.1% of the mycelial biomass growth. Valle et al. (2014) reported that under optimal conditions for laccase activity with *L. crinitus*, the concentration of 2.8 g/L of nitrogen (urea) inhibited in 72% of the mycelial biomass growth in liquid cultivation. According to D'Agostini et al. (2011), the addition of nitrogen in culture medium depends on the nitrogen source and species. They also reported that the mycelial biomass growth in the culture medium was hampered when nitrogen concentration was increased with ammonium sulfate for *P. ostreatus* and *L. edodes*, and urea for *A. brasiliensis*, both non-protein nitrogen sources. It suggests that each non-protein source need to be verified to each fungus before use, and that protein sources are far more acceptable for fungal growth.

CONCLUSIONS

The best growth range of *L. crinitus* mycelial biomass is from 31 to 34 ºC with optimal temperature peak at 32.7º C, and the best initial pH of the cultivation medium is from 4.5 to 6.5 with optimal pH peak at 6.1. Protein or non-protein nitrogen concentration is inversely proportional to the mycelia biomass growth. Nitrogen concentrations of 2.0 g/L of protein (soybean meal) and non-protein (urea) nitrogen inhibit the mycelial biomass growth in 11% and 12%, respectively, but high concentrations of 16.0 g/L of nitrogen inhibit 46% and 95%, respectively. The protein nitrogen source causes lower inhibition than the non-protein source. Overall, the fungus is robust and grows under extreme conditions of temperature and pH, but it has smaller adaptation capacity with increasing nitrogen concentrations in the cultivation medium, mainly with non-protein nitrogen.

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RESUMO: *Lentinus crinitus* é um basidiomiceto medicinal consumido por índios nativos da Amazônia brasileira. Este fungo tem sido estudado quanto ao potencial de biorremediação de metais, mas ainda carece de estudos sobre às condições básicas de crescimento. *L. crinitus* produz panepoxidona - um metabólito secundário fúngico - descrito como regulador da resposta inflamatória e imune em células animais. Este trabalho teve como objetivo avaliar as condições básicas de temperatura, pH e concentração e fonte de nitrogênio para o crescimento micelial de *L. crinitus*. O fungo foi crescido em meio agar extrato de malte a 2% (MEA), pH 5,5 e mantido entre 19 e 40 °C. Para a avaliação de pH o MEA teve o pH ajustado de 2 a 11 e o crescimento foi realizado a 28 °C. As fontes de nitrogênio estudadas foram a uréia e o farelo de soja adicionado ao MEA para obter entre 0,5 a 16 g/L de nitrogênio, pH de 5,5, cultivado a 28 ° C. A melhor faixa temperatura para o crescimento micelial foi de 31 a 34 ºC com ótimo a 32,7 º C; a melhor faixa de pH de 4,5 a 6,5 e com ótimo de 6,1. A concentração de nitrogênio proteico ou não proteico é inversamente proporcional ao crescimento do fungo. Concentrações de nitrogênio de 2,0 g/L reduzem o crescimento da biomassa micelial em 11% e 12%, respectivamente e meios com nitrogênio de 16,0 g/L reduzem o crescimento em 46% e 95%, respectivamente. O fungo é robusto e cresce sob condições extremas de temperatura e pH, mas menor adaptação em meios com alta concentração de nitrogênio, principalmente não proteico.

PALAVRAS-CHAVE: Cultivo axênico. Crescimento micelial. Condição de cultivo. Farelo de soja. *Lentinus crinitus*. Uréia.

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