

# Temperature and pH conditions for mycelial growth of *Agaricus brasiliensis* on axenic cultivation

## Condições de temperatura e pH para o crescimento micelial de *Agaricus brasiliensis* em cultivo axênico

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### Abstract

Few studies have been done to determine *Agaricus brasiliensis* Wasser et al. (*A. blazei*; *A. subrufescens*) basic mycelial growth characteristics on axenic cultivation. This study aimed to determine the optimal temperature and initial pH for mycelial growth of *A. brasiliensis* on malt extract agar medium to develop axenic cultivation techniques. Studied initial pH values for mycelial growth were adjusted to 3.0, 4.0, 5.0, 5.5, with HCl, 6.0, 7.0, 8.0, with NaOH, and again 7.0 and 8.0, with CaCO<sub>3</sub>. Studied temperatures for mycelial growth were 22 °C, 25 °C, 28 °C, 31 °C and 34 °C. It was concluded that *A. brasiliensis* can grow in axenic cultivation at temperature range from 22 °C to 34 °C, with optimal temperature range from 28 °C to 31 °C and optimal temperature value of 30.5 °C ± 0.3 °C. It also grows in initial pH range from 4.0 to 7.0, adjusted with HCl or NaOH but not CaCO<sub>3</sub>, with optimal initial pH range from 5.5 to 6.0 and optimal initial pH value of 5.56 ± 0.05. Mycelial growth is inhibited with pH of 3.0 or lower, 8.0 or higher, or when CaCO<sub>3</sub> is used to adjust pH in the substratum to 7.0 or higher.

**Key words:** Growth conditions, growth inhibition, *Agaricus brasiliensis*, *Agaricus blazei*

### Resumo

Poucos estudos foram desenvolvidos para determinar as condições básicas de crescimento micelial do fungo *Agaricus brasiliensis* Wasser et al. (*A. blazei*, *A. subrufescens*). O objetivo deste trabalho foi determinar a faixa ótima de temperatura e pH para o crescimento micelial, em agar-extrato-de-malte, de *A. brasiliensis*, visando o desenvolvimento de técnicas de cultivo axênico. Os valores de pH estudados foram 3,0, 4,0, 5,0 e 5,5, ajustados com HCl, 6,0, 7,0 e 8,0, ajustados com NaOH, e 7,0 e 8,0, ajustados com CaCO<sub>3</sub>. As temperaturas de crescimento estudadas foram 22 °C; 25 °C; 28 °C; 31 °C e 34 °C. Concluiu-se que *A. brasiliensis* cresce em uma faixa de temperatura ótima de 28 °C a 31 °C, com valor ótimo de temperatura de 30,5 °C ± 0,3 °C. A faixa de pH inicial ótimo no substrato é de 5,5 a 6,0 e o valor de pH inicial ótimo é de 5,56 ± 0,05. O crescimento do micélio é inibido com pH de 3,0 ou inferior, 8,0 ou superior, ou quando CaCO<sub>3</sub> é utilizado para ajuste do pH para 7,0 ou superior.

**Palavras-chave:** Condições de crescimento, inibição do crescimento, *Agaricus brasiliensis*, *Agaricus blazei*

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## Introduction

*Agaricus brasiliensis* Wasser et al. (*Agaricus blazei* Murrill ss. Heinemann) is a native fungus to Brazil (WASSER et al., 2002), also denoted *Agaricus subrufescens* (KERRIGAN, 2005), has been studied because of its therapeutic (MIZUNO, 2002; WATANABE et al., 2003; SOUZA-PACCOLA et al., 2004; KIMURA et al., 2004; KIM et al., 2005; SILVA et al., 2005; MANTOVANI et al., 2006) and sensory (STIJVE et al., 2002; ESCOUTO et al., 2005) properties. However, few studies describe specific techniques and parameters for *A. brasiliensis* cultivation, which is generally produced empirically or based on *Agaricus bisporus* cultivation in Brazil (BRAGA et al., 1998; EIRA; BRAGA, 2003).

Composting process is a very used technique for mushroom cultivation; however, axenic cultivation is an alternate technique due to better substrate use, medium standardization and pest control, as *Sciaridae* fly that may cause up to 50% production loss (EIRA et al., 2005). In compost cultivation the mycelial growth in the substratum must be fast to avoid the development of competing microorganisms (BRAGA et al., 1998; EIRA; BRAGA, 2003) and in the axenic cultivation it has to be fast to reduce the spawn and substratum mycelial colonization period. In this stage, it is important to control basic variables that are associated to mycelial growth as temperature and hydrogen ion concentration in solution expressed in terms of potential of hydrogen (pH).

Temperature affects enzymatic activity and vitamin synthesis and may accelerate or inhibit fungus growth (MILES; CHANG, 1997). The temperatures reported for *A. brasiliensis* growth are dissonant and vary from 18 °C to 30 °C (OKUBO; KURAMOTO; OHKUBO, 1991), 22 °C to 26 °C (IWADE; MIZUNO, 1997), 25 °C to 28 °C (EIRA; BRAGA, 2003) for compost cultivation and 30 °C for submerged cultivation (KAWAGOE et al., 2004).

Most fungi have vegetative growth at pH values from 6.5 to 6.8 and excrete extra cellular enzymes in the substrate; these enzymes present activity in a

narrow pH range, affecting fungus nutrient metabolism (MILES; CHANG, 1997). For *A. brasiliensis*, pH value of 4.5 was considered ideal for mycelial growth in submerged cultivation (KAWAGOE et al., 2004) and pH from 6.5 to 6.8 was ideal for compost cultivation (IWADE; MIZUNO, 1997). For *A. bisporus* cultivation  $\text{CaCO}_3$  or  $\text{CaSO}_4$  or both are added on compost to keep pH at 7.5 in the end of Stage II (pasteurization and conditioning) and to control  $\text{NH}_4^+$  dissociation into  $\text{NH}_3$  which is toxic to fungus (GERRITS, 1988; RINKER, 1993).

Because of dissonance for basic variables as temperature and pH ranges and lack of specific parameters for axenic cultivation of *A. brasiliensis*, the aim of this study was to determine the optimal temperature and pH for mycelial growth of *A. brasiliensis* strains in order to develop techniques for axenic cultivation.

## Material and Methods

*Agaricus brasiliensis* 97/11, 99/25, 99/26, 99/28 and 99/29 strains (COLAUTO et al., 2002), from the fungus collection of the Molecular Biology Laboratory at UNIPAR were coded as L1, L2, L3, L4 and L5, respectively. Each strain, maintained at 20 °C in malt extract agar (MEA) (24 g/L), was subcultured in Petri dishes with MEA (48 g/L) and kept in the dark at 28 °C for 10 days. Cylinders with 4mm-diameter from the edge of the mycelial growth were used as inoculum. Special care was taken to keep the cylinder mycelium in direct contact with culture medium. All experiments were quadruplicated.

For temperature study, culture medium was 48 g/L of MEA (pH 5.6) autoclaved at 121 °C for 15 min. After inoculated culture media were incubated in the dark at 22 °C, 25 °C, 28 °C, 31 °C and 34 °C. For pH study, culture medium was 48 g/L of MEA autoclaved at 121 °C for 15 min; then pH was adjusted to 3.0, 4.0, 5.0, 5.5, with HCl (1M), 6.0, 7.0, 8.0, with NaOH (1M) and another adjusted to 7.0 and 8.0, with  $\text{CaCO}_3$  (1M). All solutions were sterilized by filtration (0.22

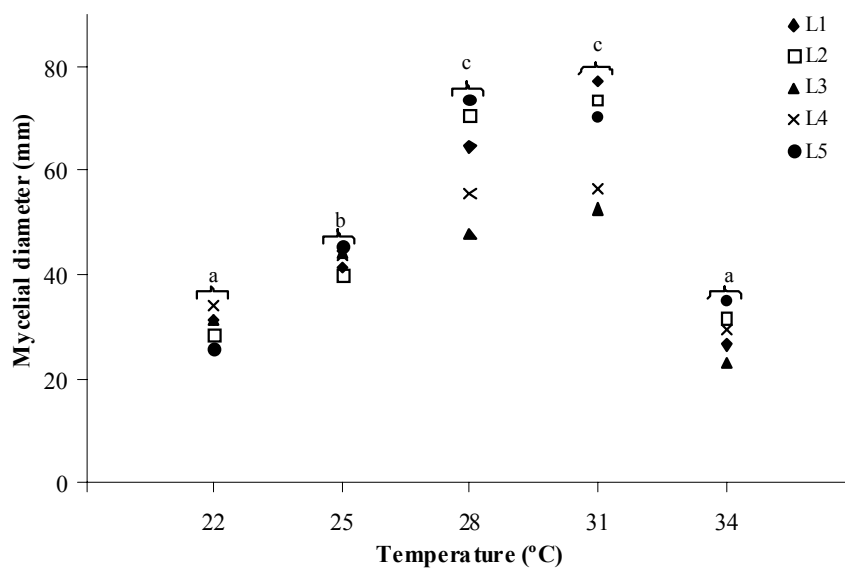
µm pore size filter). After that, culture media were inoculated and incubated in the dark at 28 °C.

For each replication, the mycelial growth was verified by calculating the average of three different measurements of the diameter (in mm) 21 days after inoculation. Four replications were made to calculate the mycelial growth diameter average for each treatment. Obtained data were evaluated using variance analysis and significant differences were determined by Tukey's test with a significance level  $p < 0.05$ . The optimal values for mycelial growth on both temperatures and pHs for each strain were determined by non-linear regression and after that an average of all strains was calculated.

## Results and Discussion

Mycelial growth results of different *A. brasiliensis* strains in function of temperature showed optimal results between 28 °C and 31 °C determined by Tukey's test (Figure). The optimal temperature for mycelial growth determined by non-linear regression was 30.5 °C for L1, 30.7 °C for L2,

30.2 °C for L3, 30.4 °C for L4 and 30.8 °C for L5. Considering all strains the mycelial optimal temperature value was 30.5 °C  $\pm$  0.3 °C. This temperature value is compatible to the ones in tropical countries, where this fungus comes from, and represents an economic advantage to mycelial growth in tropical countries. However, higher temperatures imply in more expenses to control moisture and CO<sub>2</sub> levels for fungus cultivation. Quimio, Chang and Royse (1990) reported 28 °C to 30 °C as optimal temperature for *Agaricus bitorquis* growth, a tropical basidiomycete, whereas for *Agaricus bisporus*, a fungus in which *A. brasiliensis* cultivation is based on, the recommended temperatures are from 22 °C to 25 °C. The results found in this research are similar to the ones that reported 30 °C as optimal temperature for the mycelial growth of *A. blazei* in submerged cultivation (KAWAGOE et al., 2004). Other authors as Eira and Braga (2003) and Iwade and Mizuno (1997) did not studied temperatures higher than 28 °C on compost cultivation. Thus, it was possible to verify a new range of temperature for *A. brasiliensis* growth in spawn production, axenic cultivation and possibly in compost cultivation.



**Figure.** Mycelial growth of *Agaricus brasiliensis* strains (L1 to L5) at 21 days of cultivation on malt extract agar (48 g/L) with initial pH value of 5.6 at different temperatures. Different letters represent significant differences ( $p < 0.05$ ) among temperatures.

Mycelial growth in the substrate with initial pH values of 4.0, adjusted with HCl, 7.0 and 8.0, adjusted with NaOH or CaCO<sub>3</sub>, showed different degrees of mycelial growth inhibition (Table). Unexpected the substrate adjusted with CaCO<sub>3</sub>, because of its buffering effects, inhibited mycelial growth (Table) causing possibly metabolic disorders. Thus, pH control through the traditional addition of CaCO<sub>3</sub> (BRAGA et al., 1998) affects *A. brasiliensis* growth negatively in axenic cultivation and it is likely to affect *A. brasiliensis* in other cultivation methods. This procedure of pH control with CaCO<sub>3</sub> is usual as well in spawn formulations in *A. brasiliensis* culture (BRAGA et al., 1998) and could be improved with the correct buffer and pH range in the substratum. On the other hand, the pH adjusted with NaOH allowed a natural adjustment of the substratum pH during mycelial growth besides there was a minor mycelial growth inhibition with pH of 4.0 (adjusted with HCl) and 7.0 (adjusted with NaOH) but a major inhibition when pH was 3.0 (adjusted with HCl) or 8.0 (adjusted with NaOH or CaCO<sub>3</sub>), which emphasizes the pH limits for *A. brasiliensis* growth in axenic cultivation independently of the CaCO<sub>3</sub> buffering effect for pH of 7.0 or higher in the substrate (Table). A better mycelial growth was verified with

initial pH of 5.0 but the optimal mycelial growth was between 5.5 and 6.0 by Tukey's test in axenic cultivation (Table). This pH range value for mycelial growth is compatible to the more acid Brazilian soil (MALAVOLTA, 1987), in which pH values are lower than 6.0, where *A. brasiliensis* was collected from. Thus, *A. brasiliensis*, in axenic cultivation, probably excrete extra cellular enzymes in the substrate with optimal activity in a different pH range than it was reported by Miles and Chang (1997) for most fungi (pH from 6.5 to 6.8) or by Iwade and Mizuno (1997) (pH from 6.5 to 6.8) to *A. blazei* on compost cultivation or by Kawagoe et al. (2004) (pH of 4.5) for *A. blazei* on submerged cultivation. By non-linear regression the optimal pH for mycelial growth was determined as 5.56 for L1, 5.55 for L2, 5.57 for L3, 5.59 for L4 and 5.54 for L5. The optimal initial pH considering all strains was 5.56 ± 0.05. Gerrits (1988) and Rinker (1993) reported that a pH of 7.5 was used in the substratum for *Agaricus bisporus* on compost cultivation because it makes easier control the dissociation of NH<sub>4</sub><sup>+</sup> into NH<sub>3</sub>, that is toxic to basidiomycetes. It is as well a basic and usual procedure on *A. brasiliensis* cultivation that should be reviewed after the results showed in this research, mainly when CaCO<sub>3</sub> is added in the substratum.

**Table.** Mycelial growth average of *Agaricus brasiliensis* strains (L1 to L5) at 21 days of cultivation on malt extract agar (48 g/L), kept at 28 °C with different initial pH values adjusted with HCl, NaOH or CaCO<sub>3</sub>.

pH	L1	L2	L3	L4	L5	Average (mm)	Tukey's test
3.0*	4.2	3.7	3.5	3.1	3.6	3.6	a
4.0*	69.0	67.0	43.2	50.8	61.0	58.2	c
5.0*	70.6	68.4	54.9	52.8	68.6	63.1	cd
5.5*	78.2	70.1	59.0	76.4	75.8	71.9	d
6.0**	77.5	70.5	62.2	78.0	77.5	73.1	d
7.0**	69.8	42.0	50.0	66.2	69.8	59.6	c
8.0**	6.3	8.3	9.2	5.9	6.3	7.2	a
7.0***	38.1	37.2	24.4	26.0	38.4	32.8	b
8.0***	4.8	2.3	5.7	3.6	2.9	3.9	a

\*Substratum adjusted with HCl. \*\*Substratum adjusted with NaOH. \*\*\*Substratum adjusted with CaCO<sub>3</sub>. Different letters represent significant differences ( $p < 0.05$ ) by Tukey's test.

Other usual *A. bisporus* and *A. brasiliensis* cultivation technique (BRAGA et al., 1998) is the casing layer where pH is usually adjusted to 7.5 to avoid competing fungus like *Trichoderma* (VISSCHER, 1988; FERMOR et al., 2000). Besides it is not used as substratum (COLAUTO; EIRA, 1998) the casing layer pH could affect mycelial behavior and it should be reviewed for *A. brasiliensis* axenic and compost cultivation in future researchers.

## Conclusions

*A. brasiliensis* can grow in axenic cultivation at temperature range from 22 °C to 34 °C, with optimal temperature range from 28 °C to 31 °C and optimal temperature value of 30.5 °C ± 0.3 °C. It also grows in initial pH range in the substrate from 4.0 to 7.0, adjusted with HCl or NaOH but not CaCO<sub>3</sub>, with optimal initial pH range from 5.5 to 6.0 and optimal initial pH value of 5.56 ± 0.05. Mycelial growth is inhibited with pH of 3.0 or lower, 8.0 or higher, or when CaCO<sub>3</sub> is used to adjust pH in the substratum to 7.0 or higher.

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