

Optimal Culture Conditions for Mycelial Growth of *Lignosus rhinocerus*

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Lignosus rhinocerus is a macrofungus that belongs to Polyporaceae and is native to tropical regions. This highly priced mushroom has been used as folk medicine to treat diseases by indigenous people. As a preliminary study to develop a culture method for edible mushrooms, the cultural characteristics of *L. rhinocerus* were investigated in a range of culture media under different environmental conditions. Mycelial growth of this mushroom was compared on culture media composed of various carbon and nitrogen sources in addition to C/N ratios. The optimal conditions for mycelial growth were 30°C at pH 6 and 7. Rapid mycelial growth of *L. rhinocerus* was observed on glucose-peptone and yeast extract peptone dextrose media. Carbon and nitrogen sources promoting mycelial growth of *L. rhinocerus* were glucose and potassium nitrate, respectively. The optimum C/N ratio was approximately 10 : 1 using 2% glucose supplemented as a carbon source in the basal media.

KEYWORDS : Cultural conditions, Edible mushroom, *Lignosus rhinocerus*, Medicinal mushroom, Mycelial growth

Tiger's milk mushroom (*Lignosus* spp.) is a tropical macrofungus that inhabits soil and belongs to Polyporaceae in the phylum Basidiomycota. The genus *Lignosus* comprises six species, including *L. dimiticus*, *L. ekombitii*, *L. goetzii*, *L. rhinocerus*, and *L. sacer* [1]. *L. hainanensis* was recently added when it was discovered in the province of Hainan, southern China [2].

In Malaysia, Tiger's milk mushroom (*Lignosus rhinocerus*) is the most popular and specifically sought after medicinal mushroom by the Semai aborigines upon request by urban middlemen [3]. Besides being used as tonic to maintain health, Tiger's milk mushroom is also used to treat asthma, breast cancer, cough, fever, food poisoning, and wounds.

L. rhinocerus is collected occasionally from remote regions in the states of Pahang and Perak. However, the supply of fruiting bodies and tubers of this mushroom is insufficient for local demand. Additionally, information on the culture methods of *L. rhinocerus* are scarce and no documentation for the cultural conditions are available. Hence, we obtained fundamental cultural condition information to maximize *L. rhinocerus* mycelial growth in a preliminary study to develop an artificial culture method.

Materials and Methods

Collection and isolation of *L. rhinocerus*. The fruiting bodies and tubers of *L. rhinocerus* were collected in the state of Pahang, Malaysia in June 2009. To obtain a pure *L. rhinocerus* culture, surface sterilized small pieces of pileus, stipe, and tuber were transferred to potato dextrose agar (PDA) supplemented with streptomycin (200 µg/L) and incubated in the dark for 15 days at 25°C to be used as an inoculum. Unless otherwise stated, all experiments were performed with four replications.

Culture conditions for *L. rhinocerus* mycelial growth.

pH: To screen the pH values necessary for favorable growth of *L. rhinocerus*, a 5 mm diameter plug of inoculum was removed with a cork borer from 10-day-old cultures of *L. rhinocerus* grown on PDA, placed in the center of PDA adjusted to a pH range of 4~9 with 1 N NaOH or HCl, and incubated in the dark for 10 days at 25°C. Mycelial growth was measured according to the method described by Shim *et al.* [4].

Temperature: To screen the temperature values necessary for favorable growth of *L. rhinocerus*, the fungus

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Table 1. Media composition for testing mycelial growth of *Lignosus rhinocerus*

	Media and composition (g/L)						
	PDA	YEPD	Glucose peptone	MCM	MEA	Lily	Hennerberg
Asparagine						2	
Dextrose	20	20					
Glucose			10				50
Malt extract			15	20			
Maltose					12.75	10	
Peptone		20	10	2	0.78		
Potatoes	200						
Yeast extract		10	10	2			
Dextrin NaNO ₃					2.75		2
K ₂ HPO ₄				1			
MgSO ₄				0.5		0.5	0.5
CaCl ₂							0.1
KH ₂ PO ₄				0.5		1	1
KNO ₃							2

PDA, potato dextrose agar; YEPD, yeast extract peptone dextrose; MCM, mushroom complete medium; MEA, malt extract agar.

was incubated for 10 days at five different temperatures. A 5 mm diameter plug of inoculum was removed with a cork borer from 10-day-old cultures of *L. rhinocerus* grown on PDA, placed in the center of PDA adjusted to pH 6, and incubated in the dark for 10 days at 15, 20, 25, 30, and 35°C. Mycelial growth was measured according to the method described by Shim *et al.* [4].

Culture media: Seven different culture media were prepared and screened to identify the best culture media to grow *L. rhinocerus* mycelia (Table 1). The culture media were adjusted to pH 6 before sterilization, sterilized for 15 min at 121°C, and aseptically poured into a plate. A 5 mm diameter plug of inoculum was removed from 10-day-old *L. rhinocerus* cultures grown on PDA, placed in the center of each agar plate containing one of the seven different culture media, and incubated in the dark for 10 days at 25°C. After the 10 day incubation, *L. rhinocerus* mycelial growth and density were measured.

Effect of nutrient source.

Carbon sources: To screen for the carbon sources favorable for *L. rhinocerus* mycelial growth, experiments were performed using basal media supplemented with eight different carbon sources (fructose, galactose, glucose, lactose, maltose, mannose, sorbitol, and sucrose). The basal medium was composed of peptone, 5 g; MgSO₄, 0.05 g; KH₂PO₄, 0.46 g; K₂HPO₄, 1.0 g; thiamine-HCl, 120 µg; agar, 20 g; and distilled water, 1,000 mL. Each carbon source was added to the basal medium at 0.1 M per 1,000 mL and mixed thoroughly [4]. The basal medium was adjusted to pH 6 before sterilization, sterilized for 15 min at 121°C, and aseptically poured into a plate. A 5 mm diameter plug of inoculum was removed from 10-day-old *L. rhinocerus* cultures grown on PDA, placed in the cen-

ter of basal media containing one of eight carbon sources and incubated in the dark for 10 days at 25°C. After 10 days, *L. rhinocerus* mycelial growth and density of were measured.

Nitrogen sources: To screen nitrogen source favorable for *L. rhinocerus* mycelial growth, the basal medium [5] was supplemented ten different nitrogen sources (asparagines, alanine, ammonium acetate, ammonium nitrate, glutamic acid, glutamine, glycine, phenylalanine, potassium nitrate, and urea). In addition, D-glucose was added to the basal media at 2% (w/v) and used as the carbon source for expediting mycelial growth. Each nitrogen source was added to the basal media at 0.02 M [4]. The basal media containing each nitrogen source was adjusted to pH 6 before sterilization, sterilized for 15 min at 121°C, and aseptically poured into a plate. A 5 mm diameter plug of inoculum was placed in the center of basal media containing each nitrogen source and incubated in the dark for 10 days at 25°C. After the 10 day incubation, mycelial growth and density were measured.

C/N ratio: Mycelial growth was measured in basal media that were mixed with 1, 2, 3, and 4% glucose (w/v) as a carbon source and then mixed continually with NaNO₃ as the nitrogen source. The C/N ratio (NaNO₃ : D-glucose) was adjusted to 10 : 1, 20 : 1, 30 : 1, and 40 : 1 in each media [4]. These media were adjusted to pH 6 before sterilization, sterilized for 15 min at 121°C, aseptically poured into a plate, and inoculated with a 5 cm diameter inoculum plug. The plates were incubated in the dark for 10 days at 25°C, and diameter was measured.

Statistical analysis. All data were analyzed with a one-way analysis of variance using Minitab® 15.1.1.0 (www.file-crop.com/minitab-15.1.1.0.html). A *p*-value < 0.05 was considered significant.

Results and Discussion

Culture conditions for *L. rhinoceros*. Favorable *L. rhinoceros* growth was generally obtained in the range of pH

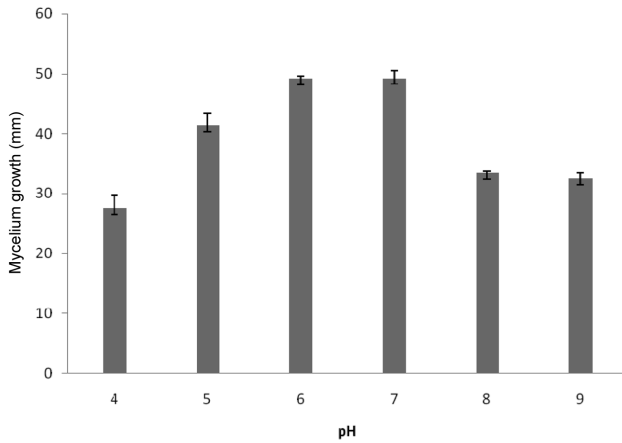


Fig. 1. *Lignosus rhinoceros* mycelial growth on potato dextrose agar at different pHs for 10 days at 25°C.

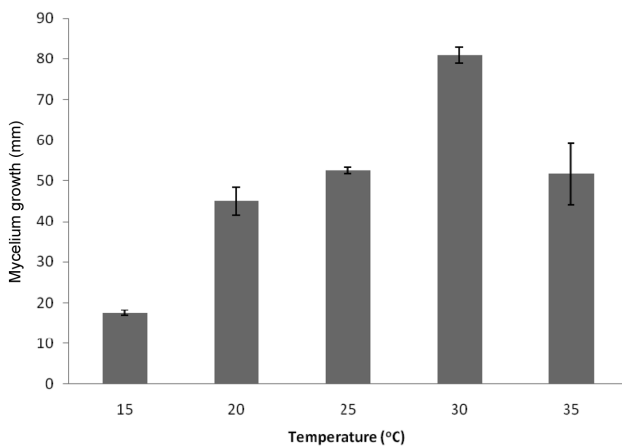


Fig. 2. *Lignosus rhinoceros* mycelial growth on potato dextrose agar for 10 days at different temperatures.

Table 2. Mycelial growth of *Lignosus rhinoceros* on various culture media

Culture medium	Colony diameter ^a (mm)	Mycelial density
Hennerberg	33.9 ± 2.21 ^b	T
Glucose peptone	68.1 ± 4.15	SC
MCM	54.4 ± 2.18	SC
PDA	36.2 ± 3.19	ST
YEPD	65.8 ± 3.16	SC
MEA	34.6 ± 3.26	T
Lily	43.4 ± 5.16	ST

MCM, mushroom complete medium; PDA, potato dextrose agar; YEPD, yeast extract peptone dextrose; MEA, malt extract agar; SC, somewhat compact; ST, somewhat thin; T, thin.

^aColony diameter was measured at 10 days of incubation.

^bValues are the average of four replicates and the SE.

5–7. The optimum pH for radial growth of *L. rhinoceros* mycelia was pH 6 and 7 (Fig. 1). However, the statistical analysis indicated no significant differences in mycelial growth between pH 6 and 7. Mycelial growth of *L. rhinoceros* was suppressed proportionally with an increase and decrease in pH. These results agreed with the optimum pH condition for *Agrocybe cylindracea* growth [6].

The temperature suitable for good *L. rhinoceros* mycelial growth was 30°C (Fig. 2), which was significantly higher than growth obtained at the other temperatures ($p < 0.05$). Growth was suppressed at temperatures > 30°C. This finding was in agreement with that of Sung *et al.* [5] who reported that *Pleurotus ostreatus* yielded its highest growth at 30°C. In addition, the current results also corroborates those of Shim *et al.* [7], in which mycelial growth of *Macrolepiota procera* gradually increased in proportion to

Table 3. Effect of carbon sources on *Lignosus rhinoceros* mycelial growth in basal medium^a

Carbon sources ^b	Colony diameter ^c (mm)	Mycelial density
Fructose	54.4 ± 1.39 ^d	SC
Galactose	20.4 ± 3.24	ST
Glucose	69.3 ± 2.35	SC
Lactose	12.4 ± 2.23	T
Maltose	24.2 ± 1.41	ST
Mannose	51.7 ± 4.38	SC
Sorbitol	18.4 ± 3.57	T
Sucrose	37.6 ± 2.28	ST

SC, somewhat compact; ST, somewhat thin; T, thin.

^aBasal medium was composed of peptone, 5 g; MgSO₄, 0.05 g; KH₂PO₄, 0.46 g; K₂HPO₄, 1.0 g; thiamine-HCl, 120 µg; agar, 20 g; and distilled water, 1,000 mL.

^bEach carbon source was added to the basal medium at 0.1 M.

^cColony diameter was measured at 10 days of incubation.

^dValues are the average of four replicates and the SE.

Table 4. Effect of nitrogen sources on *Lignosus rhinoceros* mycelial growth in basal medium^a

Nitrogen sources ^b	Colony diameter ^c (mm)	Mycelial density
Asparagine	16.3 ± 1.03 ^d	T
Glutamine	20.3 ± 0.90	ST
Alanine	24.8 ± 2.67	ST
Urea	19.0 ± 1.19	ST
Glycine	5.7 ± 1.99	T
Phenylalanine	24.7 ± 2.02	ST
Potassium nitrate	27.4 ± 3.35	ST
Ammonium acetate	23.8 ± 3.02	ST
Ammonium nitrate	22.7 ± 0.97	ST
Glutamic acid	23.7 ± 1.66	ST

C, compact; SC, somewhat compact; ST, somewhat thin; T, thin.

^aThe basal medium was composed of glucose, 20 g; MgSO₄, 0.05 g; KH₂PO₄, 0.46 g; K₂HPO₄, 1.0 g; thiamine-HCl, 120 µg; agar, 20 g; and distilled water, 1,000 mL.

^bEach nitrogen source was added to the basal medium at 0.02 M.

^cColony diameter was measured at 10 days of incubation.

^dValues are the average of four replicates and the SE.

Table 5. *Lignosus rhinocerus* mycelial growth at various carbon/nitrogen ratios (C/N) in basal medium^a

C/N ^c ratio	Colony diameter ^b (mm)			
	D-glucose concentration (%)			
	1	2	3	4
10 : 1	N.G.	1.8 ± 0.06 ^d	1.5 ± 0.08 ^d	1.5 ± 0.06 ^d
20 : 1	N.G.	N.G.	1.1 ± 0.08 ^d	N.G.
30 : 1	N.G.	N.G.	N.G.	1.2 ± 0.05 ^d
40 : 1	N.G.	N.G.	N.G.	N.G.

N.G., no growth.

^aBasal medium was composed of MgSO₄, 0.05 g; KH₂PO₄, 0.46 g; K₂HPO₄, 1.0 g; thiamine-HCl, 120 µg; agar, 20 g; and distilled water, 1,000 mL.

^bColony diameter was measured at 10 days of incubation.

^cThe ratio of NaNO₃ versus D-glucose was adjusted to 10 : 1, 20 : 1, 30 : 1, or 40 : 1, respectively.

^dValues are the average of four replicates and the SE.

a rise in temperature to 30°C. Similar to our result, growth was suppressed at 35°C.

Screening of culture media. Seven different culture media were prepared to screen for the one most suitable for *L. rhinocerus* mycelial growth (Table 1). Mycelial growth and density of *L. rhinocerus* were optimum in glucose-peptone and yeast extract peptone dextrose (YEPD), followed by mushroom complete medium (MCM) media (Table 2). However, no significant difference was observed in mycelial growth between glucose-peptone and YEPD media. Hennerberg media was the most unfavorable for radial growth of this fungus.

Effect of favorable nutrient sources.

Carbon and nitrogen sources: The carbon and nitrogen sources suitable for promoting mycelial growth of *L. rhinocerus* were glucose and potassium nitrate, respectively (Tables 3 and 4). Of the eight carbon sources tested, glucose yielded a significantly larger colony diameter of 69.3 mm but a somewhat compact mycelial density. Additionally, fructose and mannose also recorded a high radial mycelial growth rate. The combination of these three carbon sources indicates that *L. rhinocerus* prefers monosaccharides.

Among the ten nitrogen sources tested, potassium nitrate yielded the largest colony diameter of 27.4 mm with a somewhat thin mycelial density, followed by alanine and phenylalanine. These findings suggest that potassium nitrate and alanine and phenylalanine were effective for promoting mycelial growth, although growth was not significantly dif-

ferent among these nitrogen sources. These results are similar to those of Kim *et al.* [8] who found that alanine promotes mycelial growth of microfungi.

C/N ratio. The culture media that was mixed with 2% glucose as the carbon source and then adjusted to a C/N ratio of 10 : 1, showed the most favorable mycelial growth (Table 5). Generally, a gradual rise in glucose concentration seemed to suppress mycelial growth of *L. rhinocerus*. Notably, no growth occurred at a 40 : 1 C/N ratio. These results are consistent with those of Shim *et al.* [4] who found that excessive glucose concentrations suppressed mycelial growth on culture media.

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