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SHORT COMMUNICATION

Growth and antibacterial activity of *Lentinula edodes* in liquid media supplemented with agricultural wastes

Regina Hiroko Hassegawa
Departamento de Microbiologia
Instituto de Ciências Biomédicas
Av. Prof. Lineu Prestes, 1374
Ed. Biomédicas-II Cidade Universitária
05508-900, São Paulo SP, Brasil
Tel: 55 11 3727 1588
E-mail: rhassegawa@yahoo.com

Maria Catarina Megumi Kasuya
Departamento de Microbiologia
Universidade Federal de Viçosa
Av. P. H. Rolfs. s/n 36570-000 Viçosa MG
Tel: 55 031 3899 2954
Fax: 55 031 3899 2573
E-mail: mkasuya@ufv.br

Maria Cristina Dantas Vanetti*
Departamento de Microbiologia
Universidade Federal de Viçosa
Av. P. H. Rolfs. s/n 36570-000 Viçosa MG
Tel: 55 031 3899 2954
Fax: 55 031 3899 2573
E-mail: mvanetti@ufv.br

Website: http://www.ufv.br

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Antibacterial activity of *Lentinula edodes* against *Bacillus subtilis* was evaluated in cell-free filtrates obtained after growth in 14 different culture media. The highest *B. subtilis* growth inhibition was promoted by filtrates of growth media supplemented with rice bran, vermiculite or molasses. *L. edodes* dry mycelial biomass in liquid culture with 0.5% added rice bran was 3.2 mg/ml, after growth for 30 days at 25ºC without shaking, and 4.3 mg/ml under orbital shaking (150 rpm). However, antibacterial activity, detected between 20 and 24 days of incubation of stationary cultures, was absent in filtrates of aerated cultures. Temperatures of 20-25ºC enhanced both growth and antibacterial activity. Optimum pH for *L. edodes* mycelial growth was 3.0-3.5, while for production of antibacterial substance(s) it was 4.5. Our results indicated that incubation conditions that enhance mycelial growth are quite different from those necessary for production of antibacterial substance(s) by *L. edodes.*

Lentinula edodes (Berk.) Pegler, the shiitake mushroom, is one of the most widely cultivated mushrooms worldwide. The interest in shiitake cultivation is increasing because of its high nutritional value and medicinal properties, which have been acknowledged by oriental cultures, especially in China and Japan (Chang and Buswell, 1996). Interest in numerous biologically active compounds produced by this mushroom is also increasing. These include compounds with antitumour (Maeda et al. 1998; Ng and Yap, 2002), antiviral (Ngai and Ng, 2003), hypocholesterolemic (Sugiyama et al. 1995) and hypoglycemic properties (Yang et al. 2002). Antimicrobial activity has also been found in liquid cultures of *L. edodes* (Komemushiet al. 1995; Komemushiet al. 1996; Hatvani, 2001; Ishikawa et al. 2001) and chloroform, ethyl acetate or water extracts of dried mushrooms (Hirasawa et al. 1999; Ishikawa et al. 2001). Mycelial-free culture of *L. edodes* (Ishikawa et al. 2001) exhibited greater antimicrobial effect against gram-positive than gram-negative bacteria with *Bacillus subtilis* and *Staphylococcus aureus* among the most highly inhibited. Antimicrobial compounds isolated from *L. edodes* liquid cultures include lentinamicin (octa-2,3-diene-5,7 diyne-1-ol) (Komemushiet al. 1996), β-ethyl phenyl alcohol (Komemushi et al. 1996) and lentin, an antifungal

*Corresponding author

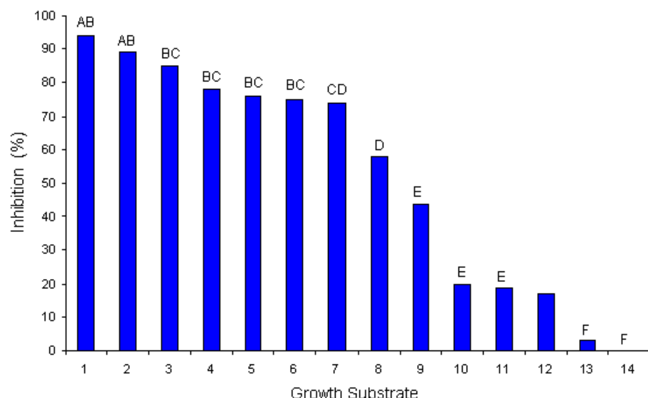

Figure 1. Inhibition of *B. subtilis* growth by *L. edodes* filtrates cultivated in culture media supplemented or not with agricultural wastes, at 25ºC. Bars followed by the same letters do not differ by Duncan test (P ≤ 0.05). 1: rice bran; 2: vermiculite; 3: sugar cane molasses; 4: oat meal; 5: mate tea; 6: MYP broth; 7: soybean bran; 8: wheat bran; 9: corn meal; 10: sugar cane bagasse; 11: YEM broth; 12: whey; 13: YMPG broth; 14: eucalyptus sawdust.

protein with molecular mass of 27.5 kDa (Ngai and Ng, 2003) .

Sawdust is the most popular basal ingredient used in synthetic substrate formulations for producing shiitake spawn. However, growth of shiitake mycelia in liquid medium is an alternative for commercial spawn production, antimicrobial and medicinal substances, and enzymes. Production of liquid inoculum of shiitake is also an alternative to the spawn used in commercial mushroom production. The advantages of liquid inoculum are its uniform distribution in the substrate and growth time reduction (Song et al. 1987).

Agricultural waste may be used as substrate to produce liquid inoculum for commercial production of shiitake. Agricultural waste bioconversion aimed at producing fungal biomass is a highly attractive alternative because, besides resulting in products of commercial interest, it reduces the amount of waste thereby minimizing pollution (Feofilova et al. 1996).

Given that few reports on growth of *L. edodes* in liquid media exist in the literature, this study was dedicated to investigating mycelial and antibacterial metabolite production by this fungus using culture broth supplemented with agricultural wastes.

MATERIALS AND METHODS

Microorganisms

L. edodes strain Le 1 was obtained from a commercial producer in Londrina, PR, Brazil and was selected by Ishikawa et al. (2001), since it presented the highest inhibitory activity towards 35 bacteria evaluated. *L. edodes* mycelia were grown on malt extract agar, in the dark, at 25ºC for 21 days. *Bacillus subtilis* strain 21, from the culture collection of the Department of Microbiology, Federal University of Viçosa (MG, Brazil) was maintained on brain heart infusion (BHI) agar slants and was used as indicator of antibacterial activity.

Culture media and growth conditions

Production of antibacterial substance(s) by *L. edodes* was evaluated in 50 ml aliquots of culture broth distributed in 125 ml Erlenmeyer flasks. The following media were used (g/l): MP broth (malt extract 30, soybean peptone 3); YMPG broth (yeast extract 3, malt extract 3, peptone 5, glucose 10); YEM broth (yeast extract 2, $CaSO_4$ 1, malt extract 10). Other culture media tested were obtained by adding 5 g of one of the following residues to YEM broth:

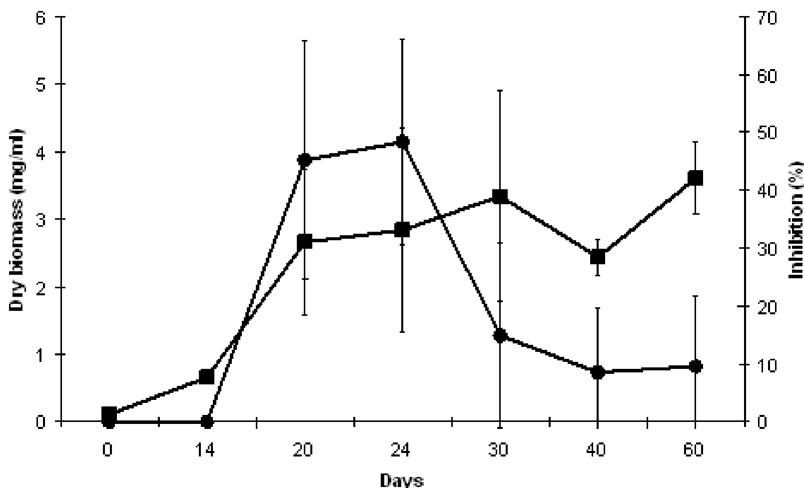

Figure 2. Dry mycelial biomass of *L. edodes* in YEM broth with 0.5% rice bran, at 25ºC under static conditions (■) and inhibition of *B. subtilis* growth by the culture filtrate of *L. edodes* (●)

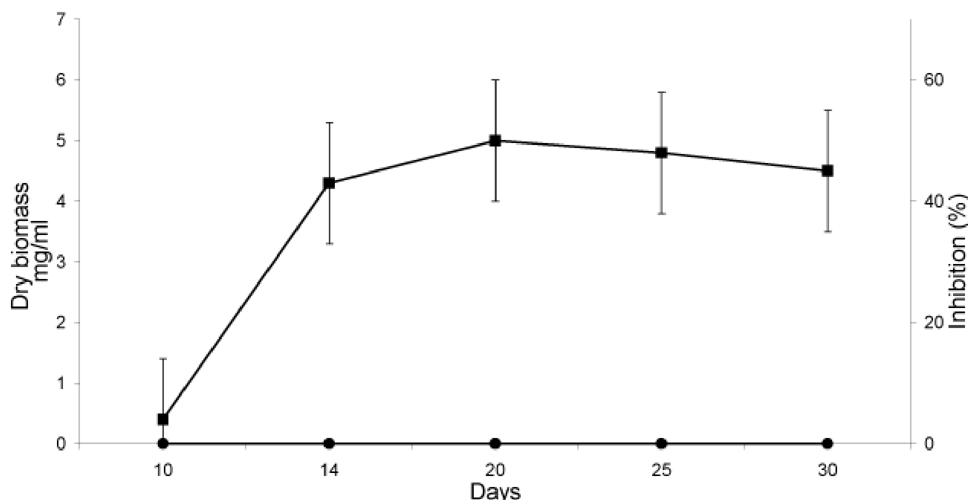

Figure 3. Dry mycelial biomass from *L. edodes* in YEM broth with 0.5% rice bran, at 25ºC under agitation of 150 rpm (■) and inhibition of *B. subtilis* growth by the culture filtrate of *L. edodes* (●).

sugar cane molasses, sugar cane bagasse, whey, rice, wheat, soybean, oat, corn, tea, eucalyptus sawdust or vermiculite. Culture broth pH was adjusted to 4.5 before sterilization (121ºC / 15 min) and was checked again at the moment of inoculation. The media were inoculated with five 7 mm diameter disks containing mycelia from the edge of *L. edodes* colonies grown on malt extract agar for 20 days at 25ºC.

Samples of the culture were filtered (Whatman nº 1, diameter 90 mm) during incubation at 25ºC for 30 days and sterilized on Millipore membranes (0.2 µm pore diameter). The filtrates were used to determine antibacterial activity against *B. subtilis* as described by Ishikawa et al. (2001). Mycelial biomass was recovered, weighed and stored at -86ºC to determine ergosterol content.

Each experiment was repeated at least tree times in duplicate. Percentage inhibition was calculated according Ishikawa et al. (2001) and statistical significance of the differences was assessed by the Duncan test ($P < 0.05$) after arcsin transformation of thee square root of percentage data.

The effect of agitation, temperature and initial pH on growth and antibacterial substance(s) production was determined in YEM broth supplemented with 0.5% rice bran and incubated at 25ºC in the dark. At each sampling time, two flasks were removed, the filtrate was obtained for antibacterial evaluation and the fresh mycelium was weighed, frozen in liquid nitrogen and stored at -86ºC to determine ergosterol content.

The effect of aeration on *L. edodes* mycelial growth and antibacterial substance(s) production was evaluated by growth in an orbital shaker (New Brunswick Scientific Co, Inc., USA) at 150 rpm and 25ºC. The fungus was also incubated at temperatures between 20 and 33ºC, and the initial pH of the media were adjusted to 3.0; 3.5; 4.0; 4.5; 5.0; 5.5; 6.0; 7.0 or 8.0, before sterilization. When necessary, the pH was adjusted with sterilized 1N NaOH or HCl, after sterilization.

Ergosterol content

Ergosterol content was determined according to the method of Richardson and Logendra (1997). Mycelial biomass was thawed at room temperature and blended for 2 min using a cell disrupter (Marconi, mod. TE 102). One gram of this suspension was added to 0.1 g of polyvinylpirrolidone (PVP) and 5 ml of 95% ethanol (Merck™) at 4ºC. After agitation, the suspension was centrifuged at 4,200 g (Sorvall® - RT6000B). The supernatant was filtered through a teflon membrane (Millipore™) with 0.50 mm pore diameter and stored at 4ºC in the dark. Ergosterol was quantified by HPLC (Shimadzu® - LC 10 A) with a Shimadzuâ reverse phase CLC-ODS column. The mobile phase was 100 % methanol (Sigma®) delivered at a flow rate of 1.0 ml min^{-1}. The elution profile was monitored at 280 nm by an UV-VIS Shimadzu® SPD-10 A detector. A standard solution was made using a 0.02% ergosterol solution (ergosta-5-7, 22-trienol) (Sigma®) diluted 1, 1:10, 1:20 and 1:100 (v/v) with methanol.

The relationship between ergosterol content and *L. edodes* dry weight was determined to be 0.37 µg/mg and this value was used to convert ergosterol content to mycelial weight.

RESULTS AND DISCUSSION

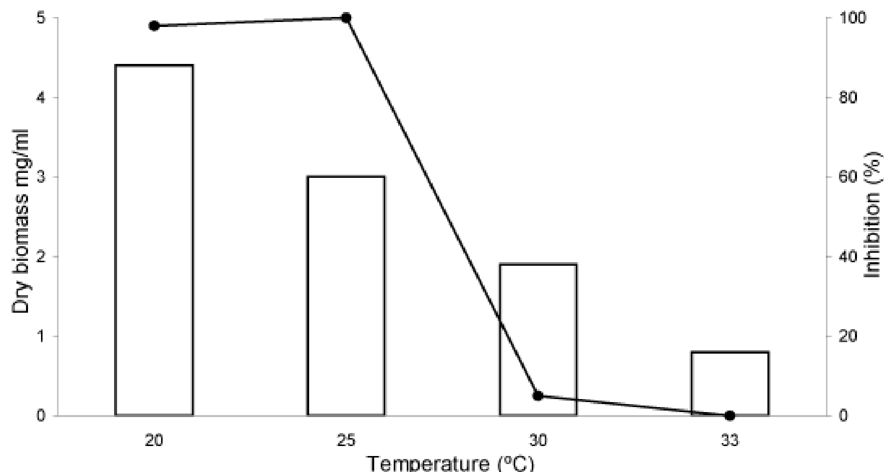

Figure 4. Dry mycelial biomass (bars) and relative inhibition of *B. subtilis* growth by *L. edodes* culture filtrate obtained at different temperature (●).

Among the substrates tested for mycelial growth of *L. edodes*, only the sawdust-containing medium was not suitable for producing antibacterial substance(s) against *B. subtilis* (Figure 1). Highest mycelial growth and antibacterial metabolite production were observed in media supplemented with rice bran, vermiculite and molasses (Figure 1). It has been demonstrated that molasses enhanced *L. edodes* biomass production and vermiculite improved growth and fructification in liquid synthetic media (Tan and Moore, 1992). Although results obtained with the aforementioned supplements did not significantly differ from each other ($P < 0.05$), rice bran was chosen for continuing this study, due to its wide availability and common use as a supplement for *L. edodes* spawn production.

The average relation between ergosterol content and mycelium dry weight of *L. edodes* was 0.37 mg/g^{-1}. Values varying between 0.12 to 0.24 mg/g^{-1} had been showed by Okeke et al. (1994) for strains of *L. edodes* cultivated in liquid medium. These authors stated that the relation between dry mycelium and ergosterol content is complex. However, ergosterol content correlated well to fungal biomass dry weight and conversion factors were 0.3-3 mg/g^{-1} biomass dry weight depending on the species (Marin et al. 2005)

Figure 2 shows mycelial growth and antibacterial activity in stationary cultures incubated at 25ºC in YEM broth with 0.5% rice bran. After 30 days of incubation, dry mycelial biomass reached 3.24 mg/ml and little increase was observed over the next 30 days (Figure 2).

The inhibitory activity against *B. subtilis* was detected in filtrates of cultures incubated at 25ºC over a narrow range of *L. edodes* growth (Figure 2). The antibacterial activity of *L. edodes* has already been reported (Komemushiet al. 1995; Pacumbaba et al. 1999; Hatvani, 2001; Ishikawa et al. 2001). However, further investigation is necessary to identify this active component. Previous studies with the strain of *L. edodes* Le1 showed that the inhibitory effect of the culture filtrate was retained in chloroform but not in aqueous extracts (Ishikawa et al. 2001) and is possibly, lentinamycin.

Growth of mycelia, appearing as pellets in the shaken cultures, increased remarkably under agitation and the biomass reached 5.0 mg/ml after 20 days (Figure 3). This value surpassed that obtained after 60 days of incubation under static conditions (Figure 2). Our result agrees with the general concept that aeration enhances fungal growth. Leatham and Griffin (1984) related that *L. edodes* growth rate in liquid media increased under agitation and the generation time was 1.5 days. Higher *L. edodes* mycelium yields was related by Song et al. (1987) when the shaking frequency was increased from 0 to150 rpm and they showed that aeration limitation in *L. edodes* cultured in Erlenmeyer flasks reduced the aerial expansion of mycelia and prevented fruiting body formation. However, Itavaara (1989) observed that the total mycelium dry weight was lower for fermented spawn cultivation when stirring was adjusted to 340 rpm and aeration to 2.0 l/min, and was highest in cultures incubated without agitation. Therefore, comparative biomass yields in stationary and shaken cultures are strain-dependent, as shown by Tan and Moore (1992). In the present study antibacterial activity was not detected in the culture filtrate after growth under agitation (Figure 3).

The optimum growth temperature for *L. edodes* was 20ºC and resulted in 4.3 mg/ml of mycelial dry biomass in YEM broth with rice bran after 24 days of incubation without agitation (Figure 4). This biomass yield was higher than those reported by Song et al. (1987) of 2.9 mg/ml dry matter at 20ºC and, approximately, 3.5 mg/ml at 25ºC upon agitation. On solid medium, *L. edodes* radial growth was

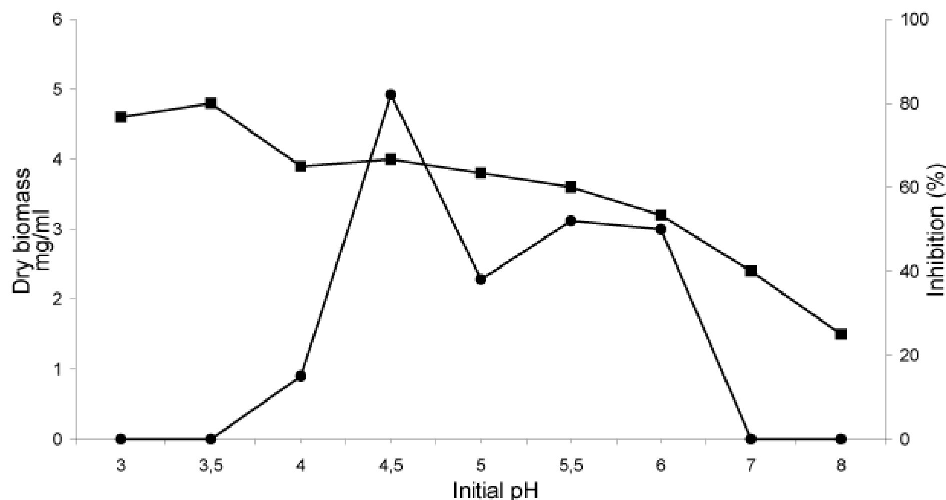

Figure 5. Dry mycelial biomass of *L. edodes* (■) and inhibition of *B. subtilis* growth (●) by culture filtrate of *L. edodes* in YEM + 0.5% rice bran with different initial pH.

highest when incubation temperature was greater than 25ºC and decreased at temperatures above 30ºC and below 20ºC (Khan et al.1991). As opposed to what was observed for aeration, the same temperature condition that enhanced mycelial growth enhanced antimicrobial activity (Figure 4).

L. edodes was able to grow over a wide range of pH while greater biomass production was detected in media with low initial pH (Figure 5), resulting in 4.6 to 4.8 mg/ml of dried biomass. This optimum pH was quite different from that observed by Song et al. (1987), who reported values between 4.3 and 4.8 in synthetic medium, under agitation. Under those conditions, dry mycelial biomass was, approximately, 4.8 mg/ml. Although pH 3.0-3.5 was most favourable for biomass production, the best antibacterial activity was observed at 4.5 (Figure 5). This result indicated that incubation conditions that enhanced growth are not the same as those that favour antibacterial activity. This result differs from those showed by Komemushi et al. (1995). These authors concluded that a pH of 4.0 was the optimum value for growth, but not for the production of antimicrobial substances against *B. subtilis* by *L. edodes*.

During mycelial growth, culture broth pH dropped from 4.0-6.0 to 3.4-3.6. This low final pH measured after mycelial growth indicates acid production by *L. edodes* is in agreement with results of other researches who found final pH varied from 3.0 to 3.8 in liquid substrates used for *L. edodes* growth (Song et al.1987; Okeke et al.1994; Rudic and Dvornina, 2001).

The results obtained in this study will be useful for evaluating liquid media to enhance *L. edodes* mycelial biomass and to evaluate substances of interest produced by this fungus, such as antibacterial compounds, enzymes and immunotherapeutic agents.

REFERENCES

CHANG, S.T. and BUSWELL, J.A. Mushroom nutriceuticals. *World Journal of Microbiology and Biotechnology*, 1996, vol. 12, no. 5, p. 473-476.

FEOFILOVA, E.P.; NEMTSEV, D.V.; TERESHINA, V.M. and KOZLOV, V.P. Polyaminosaccharides of mycelial fungi: new biotechnological use and practical implications. *Applied Biochemical and Microbiology*, 1996, vol. 32, no. 5, p. 437-445.

HATVANI, N. Antibacterial effect of the culture fluid of lentinus edodes mycelium grown in submerged liquid culture. *International Journal of Antimicrobial Agents*, 2001, vol. 17, no.1, p. 71-74.

HIRASAWA, M.; SHOUJI, N.; NETA, T.; FUKUSHIMA , K. and TAKADA, K. Three kinds of antibacterial substances from *Lentinus edodes* (Berk.) Sing. (Shiitake, an edible mushroom). *International Journal of Antibimicrobial Agents*, 1999, vol. 11, no. 2, p. 151-157.

ISHIKAWA, N.K.; KASUYA, M.C.M. and VANETTI, M.C.D. Antibacterial activity of *Lentinula edodes*. *Brazilian Journal of Microbiology*, 2001, vol. 32, no. 3, p. 206-210.

ITAVAARA, M. Comparison of three methods to produce liquid spawn for commercial cultivation of shiitake. In: *Proceedings of International Congress on the Science and Cultivation of Edible Fungi*, (12º, 1989, Braunschweig , Germany), p. 309-313.

KHAN, S.M.; MIRZA, J.H. and KHAN, M.A. Studies on shiitake mushroom (*Lentinula edodes* (Berk.) Pegler). In:

Science and Cultivation of Edible Fungi. Balkema, Rotterdam , 1991, p. 232-237.

KOMEMUSHI, S.; YAMAMOTO, Y. and FUJITA, T. Antimicrobial substance produced by *Lentinus edodes*. *Journal of Antibacterial and Antifungal Agents*, 1995, vol. 23, no. 2, p. 81-86.

KOMEMUSHI, S.; YAMAMOTO, Y. and FUJITA, T.T. Purification and identification of antimicrobial substances produced by *Lentinus edodes*. *Journal of Antibacterial and Antifungal Agents*, 1996, vol. 24, no. 1, p. 21-25.

LEATHAM, G.F. and GRIFFIN , T.J. Adapting liquid spawn *Lentinus edodes* to oak wood. *Applied Microbiology and Biotechnology*, 1984, vol. 20, p. 360-363.

MAEDA, Y.Y.; TAKAHAMA, S. and YONEKAWA, H. Four dominant loci for the vascular responses by the antitumor polysaccharide, lentinan. *Immunogenetics*, 1998, vol. 47, no. 2, p. 159-165.

MARIN, S.; RAMOS, A.J. and SANCHIS, V. Comparison of methods for assessment of growth of food spoilage moulds in solid substrates. *International Journal of Food Microbiology*, April 2005, vol. 99, no. 3, p. 329-341.

NG, M.L. and YAP , A.T. Inhibition of human colon carcinoma development by lentinan from shiitake mushrooms (*Lentinus edodes*). *Journal of Alternative Complement Medicine*, 2002, vol. 8, no. 5, p. 581-589.

NGAI, P.H. and NG, T.B. Lentin, a novel and potent antifungal protein from shitake mushroom with inhibitory effects on activity of human immunodeficiency virus-1 reverse transcriptase and proliferation of leukemia cells. *Life Science*, 2003, vol. 73, no. 26, p. 3363-3374.

OKEKE, B.C.; PATERSON, A.; SMITH, J.E. and WATSON-CRAIK, I.A. The relationship between phenol oxidase activity, soluble protein and ergosterol with growth of Lentinus species in oak sawdust logs. *Applied Microbiology and Biotechnology*, 1994, vol. 41, no. 1, p. 28-31.

PACUMBABA, R.P.; BEYL , C.A. and PACUMBABA Jr, R.O. Shiitake mycelial leachate suppresses growth of some bacterial species and symptoms of bacterial wilt of tomato an lima bean *in vitro*. *Plant Disease*, 1999, vol. 83, no. 1, p. 20-23.

RICHARDSON, M.D. and LOGENDRA, S. Ergosterol as an indicator of endophyte biomass in grass seeds. *Journal of Agricultural and Food Chemistry*, 1997, vol. 45, no. 10, p. 3903-3907.

RUDIC, V. and DVORNINA, A. Deep culturing of *Lentinula genus* (Berk.) Sing370 edible mushrooms on lignocellulose substrate. *Roumanian Biotechnology Letters*, 2001, vol. 6, p. 423-427.

SONG, C.H.; CHO, K.Y. and NAIR, N.G. A synthetic medium for the production of submerged cultures of *Lentinus edodes*. *Mycologia*, 1987, vol. 79, no. 6, p. 866-876.

SUGIYAMA, K.; ARASHI, T. and YAMAKAWA, A. Eritadenine-induced alteration of hepatic phospholipid metabolism in relation to its hypocholesterolemic action in rats. *Nutritional Biochemistry*, 1995, vol. 6, no. 7, p. 80-87.

TAN, Y.H. and MOORE , D. Convenient and effective methods for in vitro cultivation of mycelium and fruiting bodies of *Lentinus edodes*. *Microbial Research*, 1992, vol. 96, p. 1077-1084.

YANG, B.K.; KIM, D.H.; JEONG , S.C. ; DAS, S.; CHOI, Y.S.; SHIN, J.S.; LEE , S.C. and SONG, C.H. Hypoglycemic effect of a *Lentinus edodes* exo-polymer produced from a submerged mycelial culture. Bioscience, *Biotechnology and Biochemistry*, 2002, vol. 66, no. 5, p. 937-942.