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ANTIBACTERIAL ACTIVITY OF *LENTINULA EDODES* GROWN IN LIQUID MEDIUM

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ABSTRACT

The antibacterial activity of 35 isolates of *Lentinula edodes*, a shiitake mushroom, against *Bacillus subtilis* was evaluated by diffusion technique in agar with a semi-solid overlay. All isolates inhibited *B. subtilis* and the isolate Le1 promoted the formation of the largest inhibition zone. *L. edodes* Le1 also presented antibacterial activity against foodborne pathogens and food contaminant bacteria, particularly Gram-positive species. The antibacterial activity of the culture filtrate after 18-25 days of cultivation of *L. edodes* in broth at 25ºC was high. The inhibitory activity was observed only in the organic layer when the culture filtrate was partitioned between ethyl acetate and water, suggesting that the inhibitory substances have low polarity. The silica gel thin-layer zone at *Rf* values of 0.63-0.80, developed in chloroform - acetone - ethyl acetate - methanol = 40:5:5:2, was responsible for the antibacterial activity against *B. subtilis*. The inhibitory activity of *L. edodes* was detectable in the culture filtrate after heat treatment at 100ºC for 10 min and after storage at 4ºC for 120 days.

Key words: antibacterial activity, *Lentinula edodes*, shiitake

INTRODUCTION

The shiitake mushroom, *Lentinula edodes* (Berkeley) Pegler, is the most commercially important mushroom grown on wood (23). It is considered a delicacy and an essential ingredient for many Japanese and Chinese dishes (24). The cultivation of shiitake is expanding in Brazil, using eucalypt logs, particularly in the South and Southeastern regions, due to favorable climatic conditions. Shiitake is a model among functional mushrooms for extensive research of its bioactivity leading to the isolation of pure compounds which have pharmaceutical status (3). Medicinal properties such as anti-tumor (5,15), anticarcinogenic (19), anti-viral (12,17,21), preventive blood pressure increase in hypertension cases (9), and hypocholesterolemic (4,20) have been attributed to active substances extracted from *L. edodes*. Antibacterial, antifungal and antiviral activities were also reported (8). The antimicrobial activity of *L. edodes* against *Trichoderma*, the main genus detected in damaged bedlog, was related by Tokimoto *et al.* (22), who verified the increasing production of antifungal substances by shiitake in the presence of this contaminant. The inhibitory activity in *L. edodes* culture broth against bacteria and fungi has also been observed (13,14).

In this study we verified the inhibitory activity of *L. edodes* isolates against *B. subtilis* and on some foodborne bacteria.

MATERIALS AND METHODS

Organisms

Sixteen of thirty five strains of *L. edodes* used in this study were isolated from mushrooms of shiitake growers in Paraná, São Paulo and Minas Gerais states in Brazil. The remaining cultures were kindly provided by the collection of Botanic Institute of São Paulo, Brazil. The stock cultures were kept in tubes with potato dextrose agar (PDA, Merck). Mycelium of each stock was cultured at 25ºC on the surface of malt extract agar (Difco) in 9cm diameter Petri dishes. After 15 days, mycelial disks were punched out with a 7mm diameter cork borer. Foodborne pathogenic and spoilage bacteria strains, listed in Table 1, were from the culture

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collection of the Food Microbiology Laboratory at the University Federal of Viçosa (MG), Brazil. Stocks were maintained on Brain Heart Infusion (BHI, Merck) slants at 4ºC.

Antibacterial activity of *L. edodes*

The evaluation of inhibitory activity of 35 cultures of *L. edodes* was performed according to the modified method of Spelhaug and Harlander (18). The mycelial disks (7mm diameter) of fungal strains were transferred onto malt extract agar. After 3 days incubation at 25ºC, 7ml of semi-solid BHI inoculated with about 10^5 CFU/ml of *B. subtilis* was carefully poured on the plate surface as an overlay. *B. subtilis* was chosen as indicator bacterium because, in preliminary tests, its growth was markedly inhibited by the presence of *L. edodes*. Furthermore, this bacterium was also used by Komemushi *et al.* (13) to test the antimicrobial activity of *L. edodes*.

The Petri dishes were allowed to stand for 8 hours at 4ºC (6) for diffusion of the metabolites of *L. edodes* culture to the BHI overlay and then incubated at 37ºC for 18hs. Antibacterial activity was determined by measuring the radius of the clear inhibition zone around each mycelial disk. Four replicates were made for each *L. edodes* culture. The strain of *L. edodes* that showed the highest antagonistic activity against *B. subtilis* was selected for evaluation of the antibacterial activity against the foodborne bacteria listed in Table 1.

The antibacterial activity of *L. edodes* was either evaluated on liquid culture filtrate or on mycelium biomass. The fungi were cultured in 250ml Erlenmeyer flasks containing 50ml of malt extract broth. After 30 days incubation at 25ºC, as static cultures, the mycelial biomass was retained on a sieve, and the broth was sterilized by filtration through a 0.45µm pore-size membrane. The mycelium extract was prepared according to Kasuya (10). The antibacterial activity was determined on 3ml BHI broth dispensed in screw cap tubes inoculated with about 10^5 CFU/ml of a 18hs culture of *B. subtilis*. One milliliter of *L. edodes* culture filtrate or extract of mycelial was added, and the bacterial growth was determined by measuring the optical density (OD) at 600nm and by the number of viable cells on BHI agar inoculated with a spiral plater (Spiral Biotech, Inc. mod. 4000, Bethesda, MD). In the control tube, 1ml of sterilized water was added instead of the filtrate or extract. Percentage of inhibition was calculated using the formula: [(OD control - OD filtrate or extract)/OD control] x 100. The experiment was done in triplicates.

The effect of incubation time upon the antibacterial activity of *L. edodes* was tested using the filtrate obtained from cultures incubated in malt extract broth, at 25ºC over 40 days. Periodically, three flasks were removed to evaluate spectrophotometrically the *B. subtilis* inhibition in BHI broth.

Extraction of antibacterial substance of filtrate broth was performed according to the procedure of Kasuya *et al.* (11). Briefly, *L. edodes* was grown in 50ml malt extract broth for 30 days at 25ºC. The constituents in the culture filtrate were partitioned between ethyl acetate and water. The inhibitory activity of organic and aqueous fractions was evaluated using paper disks imbibed with sample solutions. *B. subtilis* was used as the indicator bacterium and was spread over plate surface before the filter-papers were placed. The plates were maintained in a refrigerator for 8h for diffusion of substances from paper disk to agar and then, incubated at 37ºC for 18hs. The radius of the inhibitory zone was recorded. Control tests with solvent gave no zones of inhibition.

Table 1. Radius of the inhibition halo of foodborne pathogens and food contaminant bacteria caused by the culture filtrate of *Lentinula edodes* Le1.

Bacteria		Strain	Inhibition halo (mm)
Gram-positive			
Bacillus cereus		F 4433	12
		F 4810	11
B. subtilis		LMA 0011	28
Listeria innocua		12570	8
		L6A	4
L. monocytogenes		ATCC 7644	4
		Scott A	3
	(1)		0
	(1)		0
	(1)		13
Staphylococcus aureus	(2)		13
	(3)		15
	(4)		19
S. epidermidis		ATCC 12228	27
Gram-negative			
Acinetobacter	(2)		0
Citrobacter amaloni	(3)		0
Enterobacter cloacae		ATCC 23355	0
Escherichia coli		ATCC 25922	0
	(4)		0
Enteropathogenic *E. coli*			0
Klebsiella pneumoniae		ATCC 13883	4
Proteus mirabilis		ATCC 25933	4
Pseudomonas aeruginosa	(2)		0
P. maltophila	(2)		6
Salmonella anatum		9021	0
S. galinarium		LMA 0021	0
S. saintpaul		LMA 0022	0
Salmonella sp.	(3)		0
Serratia marcescens	(3)		0
Shigella sonnei	(4)		0
Yersinia enterocolitica	(4)		5

(1) Isolated from foods; (2) Isolated from food handler; (3) Isolated from food implement surface; (4) Isolated from food utensils.

The heat stability of antibacterial substances from *L. edodes* was evaluated by using samples heated at 100ºC for 10, 20, 30 and 60min. After heat treatment, the filtrate was quickly cooled in an ice bath. The control filtrate was left at room temperature. The inhibitory activity against *B. subtilis* was determined on spectrophotometer, as described before.

TLC-bioautography

Pre-coated glass plates of Silica Gel 60 F_{254} (Merck) were used to detect the ethyl acetate soluble antibacterial compounds in the culture filtrate. An aliquot of ethyl acetate extract equivalent to 581.24mg of mycelial dry matter was charged on the plate and developed in a solvent system of chloroform - acetone - ethyl acetate - methanol = 40:5:5:2. After development, the solvents were evaporated under reduced pressure and the bands of quenching and fluorescent compounds were observed under UV light at 254 and 366nm, respectively. For bioautography, 40ml of semi solid BHI inoculated with *B. subtilis* was sprayed to TLC plates. After 1h at refrigerator temperature, the plates were incubated for 15hs at 37ºC in a box lined with wet filter paper to maintain high humidity. Application of a superficial layer of aesculin lead to better observation of inhibition zone (25).The plates were incubated for an additional 4hs to allow hydrolysis of aesculin by *B. subtilis*.

RESULTS AND DISCUSSION

All isolates of *L. edodes* inhibited the growth of *B. subtilis*, determined by the formation of an inhibition halo (Fig. 1). However, this antimicrobial activity varied markedly. The isolate Le1 promoted the formation of the largest inhibition halo and was selected for further study.

The isolated Le1 presented antibacterial activity against 8 species out of 20 foodborne pathogens and food contaminants, mainly on Gram-positive bacteria (Table 1). Coletto (2) observed the antimicrobial activity of *L. edodes* upon Gram-positive bacteria as *Staphylococcus aureus* and *B. subtilis*, but not upon *Escherichia coli*. However, Herrmann (7) and Komemushi *et al.* (13) showed inhibitory effect upon Gram-positive and negative bacteria. In our study, *B. cereus*, *B. subtilis*, *S. aureus*, and *S. epidermidis* presented high sensitivity to metabolic compounds of *L. edodes*. *B. cereus* and *S. aureus* are widely recognized as important foodborne pathogens and the potential of its inhibition presented by *L. edodes* may receive more attention. Although the *in vivo* effect of antibacterial substance of *L. edodes* was shown to be due to the induction complement C3 elevation that increased the resistance to infection (16), the results presented here suggest that some direct antibacterial effect would occur and should be further exploited.

The inhibition of *B. subtilis* growth observed in the broth containing mycelium culture filtrate, and the lag phase detected in the broth with mycelium extract (Fig. 2) indicate that the substance(s) of *L. edodes* with potential inhibitory effect is(are) both extra and intracellular. Similar results were also observed by Komemuschi *et al.* (13).

The inhibition of *B. subtilis* growth was observed when mycelial filtrate was added to BHI broth (Fig. 2). The number of viable cells of *B. subtilis* after 12hs at 37ºC remained quite

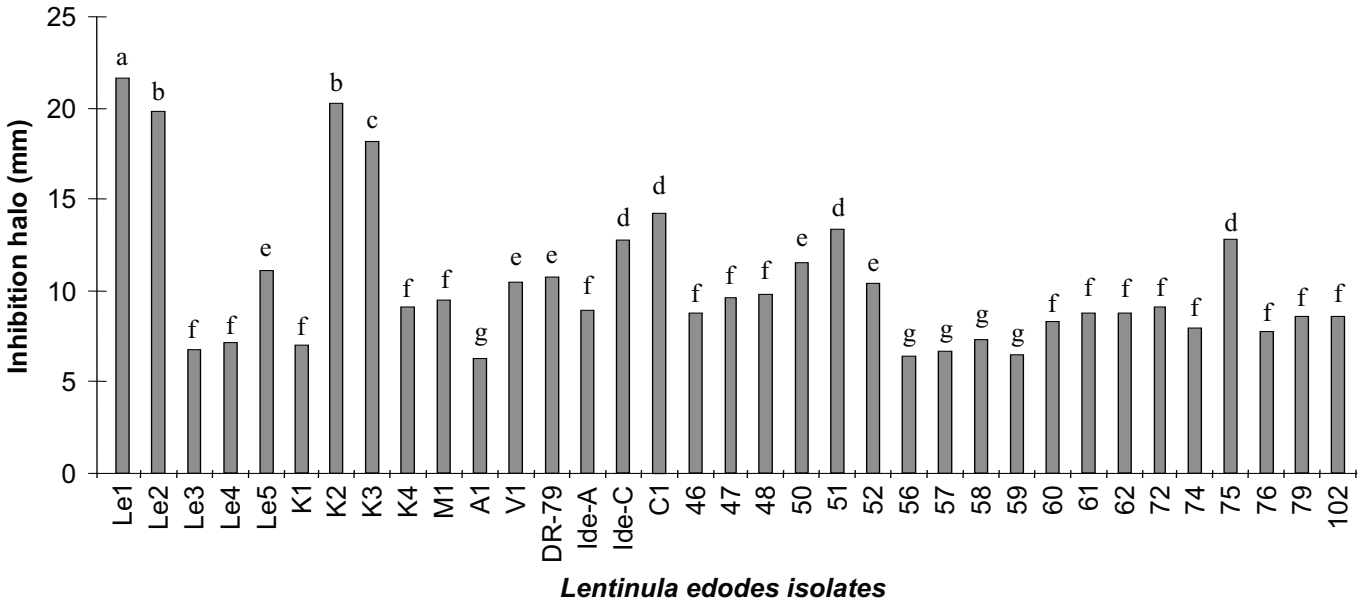

Figure 1. Inhibition halo (mm) of *Bacillus subtilis* by *Lentinula edodes* isolates. Bars followed by the same letter do not differ by Scott Knott test ($P \leq 0.05$).

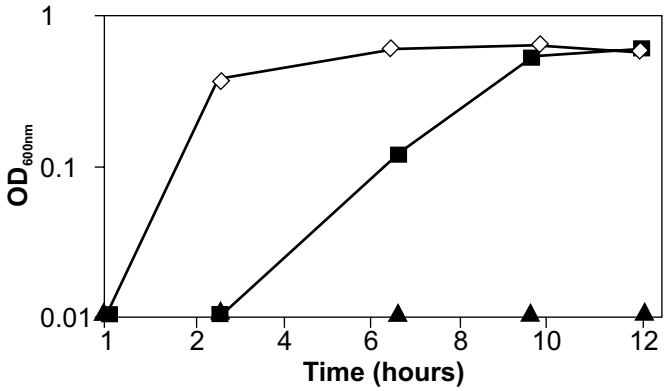

Figure 2. Growth of *Bacillus subtilis*, at 37ºC in BHI, alone (-◇-) or added with mycelium culture filtrate (-▲-) or mycelium extract (-■-) of *Lentinula edodes*.

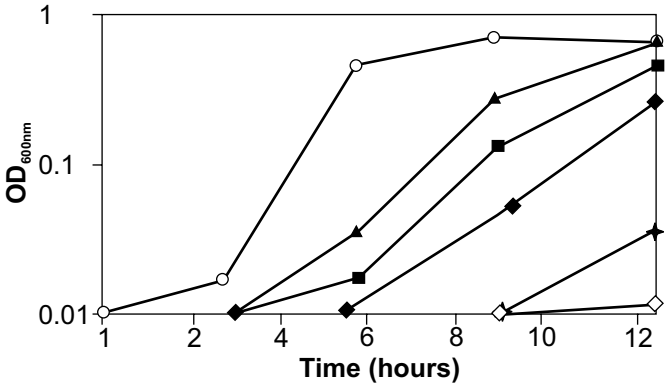

Figure 3. Effect of heat treatment of culture filtrate of the *Lentinula edodes* on the growth of *Bacillus subtilis*: control (BHI broth without filtrate) (○); filtrate without heat treatment (◇); filtrate heated at 100ºC/10 min. (✧); 100ºC/20min. (◆); 100ºC/30min. (■); 100ºC/60min. (▲).

constant, 10^3 CFU/ml, compared to the variation of 5 log cycles observed in the control, during the same time of incubation. This suggested that the effect of inhibitory substance was bacteriostatic.

The antibacterial activity of the mycelium filtrate was dependent on the age of *L. edodes* culture. The highest inhibition was observed between 18 to 25 days of cultivation in malt extract broth at 25ºC, when the growth inhibition was, respectively, 84 and 100% after 12hs incubation (data not shown).

The inhibitory activity was observed in the organic layer, after extraction of the culture filtrate with ethyl acetate, indicating that inhibitory activity is due to substances with low polarity. The results observed with the water and ethyl acetate soluble fractions showed the inhibitory activity only around the paper disks treated with the ethyl acetate extract. Antifungal substances produced by *L. edodes* have been identified as straight-chain alcohol with 8-9 carbons, having double and triple bonds (22). One of these substances was lentinamycin, extracted and identified by Bew *et al.* (1). Lentinamycin was the main cause of the antimicrobial activity of the culture filtrate of *L. edodes* upon bacteria, filamentous fungi and yeasts (14).

The heating of *L. edodes* filtrate at 100ºC for 10min did not cause a significant loss of antibacterial activity against *B. subtilis* (Fig. 3). However, the increase of the exposition time to 20, 30 and 60min resulted in 43, 72 and 100%, of activity reduction, respectively, when compared with that of filtrate maintained at room temperature.

In the bioautography on TLC, an inhibition activity against *B. subtilis* was observed between *Rf* 0.63 and 0.80. The large range of *Rf* observed may be due to the diffusion and/or volatile property of the active principles. The presence of inhibitory substance in the culture broth of *L. edodes* against *Aspergillus ochraceus,* after fractionating on TLC with benzene - ethyl acetate = 75:25, was also observed by Komemushi *et al.* (13), and the inhibitory zone was around *Rf* 0.60.

Considering the potential use of natural antimicrobial compounds in food processing, at present we are working on the isolation and identification of the major antimicrobial agents produced by *L. edodes*.

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RESUMO

Atividade antibacteriana de *Lentinula edodes* cultivado em meio líquido

A atividade antibacteriana de 35 isolados de *Lentinula edodes* (shiitake) contra *Bacillus subtilis* foi avaliada pela técnica de difusão em sobrecamada de ágar semi-sólido. Todos os isolados avaliados inibiram o crescimento de *B. subtilis* sendo o isolado Le1 o que apresentou maior halo de inibição. *L. edodes* Le1 também inibiu o crescimento de bactérias patogênicas e deterioradoras de alimentos, especialmente as Gram-positivas. Maior atividade antibacteriana do filtrado da cultura de *L. edodes* em meio líquido foi observada entre 18-25 dias de incubação, a 25ºC. O fracionamento do filtrado da cultura com acetato de etila e água permitiu evidenciar a atividade antagonista somente na fase orgânica, sugerindo que as substâncias inibitórias apresentam baixa poloridade. A cromatografia em camada fina,

com o sistema de solventes clorofórmio - acetona - acetato de etila - metanol = 40:5:5:2, definiu a região de Rf entre 0,63-0,80 como a que apresenta atividade antibacteriana contra *B. subtilis*. A atividade inibitória de *L. edodes* foi detectada em filtrado da cultura após o tratamento térmico de 100ºC por 10 minutos e após estocagem a 4ºC por 120 dias.

Palavras-chave: atividade antibacteriana, *Lentinula edodes*, shiitake

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