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Short communication

Antibacterial effect of the culture fluid of *Lentinus edodes* mycelium grown in submerged liquid culture

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Abstract

The antimicrobial activity of the culture fluid of *Lentinus edodes* mycelium grown in submerged liquid culture was tested against some common bacterial species and *Candida albicans*. The mycelium-free culture fluid was bacteriostatic against *Streptococcus pyogenes*, *Staphylococcus aureus* and *Bacillus megaterium*. The substance responsible for the activity was heat-stable, could be extracted with chloroform and had a molecular weight under 10 000. These characteristics suggested that the component might be lenthionine, an antibacterial and antifungal sulphur-containing compound. The culture fluid was less toxic to human tissue culture cells than to microbes. The antibacterial activity and toxicity could not be attributed to the same component.

Keywords: Antibacterial effect; Culture fluid; *Lentinus edodes*; Shiitake mushrooms; Submerged liquid culture

1. Introduction

The importance of the Shiitake mushroom (*Lentinus edodes*), the second most popular edible mushroom in the global market, is attributed not only to its nutritional value, but also to possible medical and food industrial applications. Lentinan (a polysaccharide isolated from the fruiting body) acts as a host defence potentiator. Besides its anti-tumour activity, it has been demonstrated to increase the host resistance to bacterial and viral infections [1]. Lenthionine, a sulphur-containing compound, has antibacterial and antifungal activity [2,3], and bis[(methylsulfonyl)methyl]disulphide, a derivative of lenthionine, has strong inhibitory effects against *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli* [4]. The chloroform and ethylacetate extracts of the dried mushroom have antibacterial activity against *Streptococcus mutans* and *Prevotella intermedia* [5]. Both fruiting body and the mycelium contain compounds with wide-ranging antimicrobial activity [1]. Several fractions of LEM (an aqueous extract of the *L. edodes* mycelium and its solid culture medium) have immunoactive properties such as the induction of interferon in vitro [6] and in vivo [7], inhibition of the infectivity and cytopathic effect of human immunodeficiency virus [8,9] and blockade of the release of herpes simplex virus type 1 from tissue culture cells [10].

The antibacterial activity of the supernatant of the submerged liquid culture was investigated in this study.

2. Materials and methods

2.1. Strains

Strains of *Campylobactor jejuni*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Bacillus megaterium*, *Pseudomonas fluorescens*, *Micrococcus luteus* and *Klebsiella pneumoniae* were from the collection of the Institute of Public Health and Medical Officer Service, Szeged, Hungary. The *L. edodes* strain 610 was from our institute collection.

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2.2. Culture conditions

2.2.1. Growth of tested micro-organisms

All bacterial strains were cultured in Nutrient Broth (Oxoid Ltd.) at 37°C, with the exception of *C. jejuni*, which was grown under anaerobic conditions in a special broth containing peptone (10 g/l), Lab Lemco powder (10 g/l, Oxoid), NaCl (5 g/l), casein hydrolysate (3 g/l), active carbon (4 g/l), $FeSO_4$ (0.25 g/l), sodium pyruvate (0.25 g/l), sodium deoxycholate (1 g/l) and NaOH (0.28 g/l). *C. albicans* was grown in liquid medium containing glucose (40 g/l), peptone (10 g/l) and yeast extract (2 g/l). The agar plates used for viable counts contained the same nutrients and agar (30 g/l).

2.2.2. Growth of L. edodes mycelium

L. edodes mycelium was grown at 25°C in submerged liquid cultures. The liquid medium (pH 6.6) contained glucose (20 g/l), peptone (10 g/l) and yeast extract (2 g/l). One hundred millitres of the medium in a 250 ml Erlenmeyer flask was inoculated with agar plugs covered by the mycelium. An inoculum of 50 ml of the 23 day-old shaken culture (160 rpm) started the fermentation in a Wheaton Celstir flask (500 ml medium inoculated in a 1000 ml flask). The culture fluid was harvested after 7 weeks of fermentation (25°C, with stirring at 180 rpm), when the mycelium concentration was approximately 4.5 g wet weight/l, i.e. 1 g dry weight/l. The pH had decreased to 3.6 by the end of the fermentation process.

The *L. edodes* strain was maintained on agar plates with the same composition as that of the liquid medium and agar (30 g/l).

Before use in experiments, the pH of the culture fluid was adjusted to 7.2 with 1 N NaOH to avoid possible inhibition of microbial growth by acidic conditions.

2.3. Heat treatment

The culture fluid was treated at 60°C for 30 min and at 100°C for 5 min.

2.4. Filtration

The culture fluid (2 ml) was filtered by ultrafiltration through a 10 000 MW cut-off membrane (Centricon YM-100, Amicon, Millipore). After filtration, the retained fraction (50 μl) was diluted to the starting volume (2 ml) with deionized water.

2.5. Extraction of the culture fluid

1 ml culture fluid was extracted three times with 3×1 ml chloroform:methanol (9:1) solution. The chloroform fraction was evaporated and the extracted materials were dissolved in the original volume (1 ml) of deionized water. The aqueous fraction was retained.

2.6. Assay for antibacterial activity

The initial samples containing the culture fluid or its different fractions were diluted 10-, 20- or 30-fold with physiological saline after aseptic filtration using a syringe filter (Puradisc 13 PVDF, Whatman). Since the culture fluid contained abundant residual nutrients and especially glucose (unpublished results, [11]), the initial samples had to be completed with the appropriate amount of the medium used for the cultivation of *L. edodes* mycelium. These samples (900 μl final volume) were inoculated with 100 μl of the 10-fold dilution of the 24 h-old bacterial culture. The antibacterial activity was measured via determination of the living cell number of the samples. 50 μl of the 10^2-fold and 10^3-fold dilutions of the samples were plated on agar plates and the number of colony-forming units (CFU) was counted after incubation of the plates at 37°C for 24 h.

2.7. Assay for toxicity

The cell culture used in the in vitro toxicity test was a continuous culture of a human amnion epithelial cell line (WISH) grown as a monolayer under 5% CO_2 at 37°C, in a medium (Basal Eagle's MEM, Glasgow modification) supplemented with 5% inactivated foetal calf serum. Cells growing for 24 h in 96-well microplates (1.6×10^4 cells per well) were treated with the culture fluid and its different fractions: 100 μl of culture medium in the wells contained the samples in two-fold serial dilutions. The toxic effect was checked after incubation at 37°C for 24 h.

3. Results

3.1. Antibacterial activity of the culture fluid

Treatment with *L. edodes* culture fluid was effective against three of the bacterial strains tested. The data relating to the antibacterial activity are summarised in Table 1; each result is the mean of three replicates. *S. pyogenes* proved most sensitive. With the exception of *B. megaterium* at 10-fold dilution, growth occurred in every sample, but the rate was lower in the samples containing culture fluid and suggesting a bacteriostatic effect. The inhibitory effects of the 20- and 30-fold dilutions were similar for all three strains.

The culture fluid showed poor activity against *C. albicans* and had no effect on *E. coli*, *P. fluorescens*, *M. luteus*, *K. pneumoniae* and *C. jejuni*.

3.2. Antibacterial activities of the preparations and fractions from the culture fluid

As *S. pyogenes* was the most sensitive of the organisms tested, the antibacterial activity of the fractions against this bacterium was investigated.

None of the heat treatments decreased the antibacterial efficiency of the culture fluid. The active substance responsible was in the filtrate (MW < 10 000) after ultrafiltration. There was a decrease in viable count in samples containing the filtrate in 10-fold dilution. Bacterial growth was not affected by the retained fraction (MW > 10 000) or the aqueous fraction. The chloroform fraction contained the active component. The heat-treated culture fluid, the filtrate and the chloroform fraction exhibited the same bacteriostatic effect against *S. pyogenes* as that in the sample containing the original culture fluid.

3.3. Toxicity

At eight-fold and 16-fold dilutions of the culture fluid, 50% and 25%, respectively, of the tissue culture cells were dead. There was no cell destruction by the 32-fold dilution. The bacteria were more sensitive to the treatment than were the human tissue culture cells, especially at lower concentrations.

The components responsible for the toxicity were present in the filtrate and the aqueous fraction of the original culture fluid. The chloroform fraction was not toxic: all the cells remained viable even at a two-fold dilution of the preparation.

4. Discussion

Earlier work showed that *L. edodes* contains several compounds that exert inhibitory effects on a wide range of microbes [3–5]. The results of our experiments showed that the sterile, mycelium-free culture fluid fermented by the mycelial biomass in a submerged culture had a similar effect. The culture fluid was less toxic to human tissue culture cells than to microbes. The antibacterial activity and the toxicity cannot be attributed to the same component of the culture fluid, since the chloroform fraction contained the bacteriostatic compound and the aqueous fraction had some toxic effect, but did not affect bacterial growth.

Lenthionine [2,3,5] is detectable in the fruiting body of *L. edodes* and in the mycelium biomass produced in submerged culture [11]. It is probable that the active component of the culture fluid in our extraction was lenthionine. Further investigation of the component is needed for confirmation. Addition of sulphur-containing substrates to the liquid medium might enhance production of the antibacterial compound.

Beneficial volatile compounds of the Shiitake mushroom, such as antimicrobial agents, aroma and flavours, are present in the mycelium [1,11]. The biomass produced in submerged liquid fermentation processes could therefore be used as a human food supplement, as an animal feed supplement or as a flavouring agent. The culture fluid by-product of the process, or its purified fractions, might be used instead of chemical agents used to disinfect surfaces or as preservative agents in food packaging technology, in view of the low toxicity. If the preservative agent does not come into direct contact with the food, or if the quality of the food product is not influenced by the low pH value, the pH would not have to be raised and the antibacterial activity could be more efficient.

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Table 1
Antibacterial activity of *L. edodes* culture fluid

	Initial viable count (CFU/ml)	Viable count after 24 h (CFU/ml)			
		10-fold dilution	20-fold dilution	30-fold dilution	control
Bacillus megaterium	4.4×10^4	0 (0%)[a]	2.3×10^6 (45%)	2.6×10^6 (51%)	5.1×10^6 (100%)
Staphylococcus aureus	1.2×10^4	2.1×10^4 (11%)	8.0×10^4 (40%)	1.2×10^5 (60%)	2.0×10^5 (100%)
Streptococcus pyogenes	3.3×10^4	6.0×10^4 (5%)	2.4×10^5 (19%)	2.2×10^5 (17%)	1.3×10^6 (100%)
Candida albicans	3.1×10^4	6.8×10^5 (43%)	8.2×10^5 (51%)	1.5×10^6 (94%)	1.6×10^6 (100%)

[a] The numbers in parentheses indicate the living cell number as a percentage of that in the control sample.

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