Oxalic Acid from Lentinula edodes Culture Filtrate: Antimicrobial Activity on Phytopathogenic Bacteria and Qualitative and Quantitative Analyses

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Abstract The culture filtrate of Lentinula edodes shows potent antimicrobial activity against the plant pathogenic bacteria Ralstonia solanacearum. Bioassay-guided fractionation was conducted using Diaion HP-20 column chromatography, and the insoluble active compound was not adsorbed on the resin. Further fractionation by high-performance liquid chromatography (HPLC) suggested that the active compounds were organic acids. Nine organic acids were detected in the culture filtrate of L. edodes; oxalic acid was the major component and exhibited antibacterial activity against nine different phytopathogenic bacteria. Quantitative analysis by HPLC revealed that the content of oxalic acid was higher in the water extract from spent mushroom substrate than in liquid culture. This suggests that the water extract of spent L. edodes substrate is an eco-friendly control agent for plant diseases.

Keywords Antibacterial activity, Oxalic acid, Qualitative and quantitative analyses

Lentinula edodes exhibits properties such as high nutritional value and medical efficacy and has been shown to have antitumor, immunopotentiation, and hypoglycemic properties [1-3]. In addition, the antimicrobial compound lenthionine and its derivative show inhibitory effects on bacterial and fungal species and have been isolated from fruiting bodies and mycelia [4, 5]. Additionally, the antibacterial effects were tested in mycelium-free culture fluid produced from a submerged liquid culture of L. edodes [6]. In Korea, the yield of L. edodes (Pyogo mushroom in Korea) is estimated to be approximately 37,000 tons per year, making it an economically important mushroom. The mushroom is mainly cultivated on tree logs, but sawdust bag cultivation has recently rapidly increased each year, accounting for more than 50% of the phyogo mushroom cultivation in Korea because of its high yield. Mushroom growers must dispose of the spent mushroom substrate (SMS), a by-product of bag cultivation. Based on its culture characteristics, SMS is very similar to a type of solid-state fermentation [7] used to produce large quantities of antibiotics. Thus, SMS may contain a variety of bioactive compounds with antimicrobial activity and may be a good material for extracting antimicrobial compounds. It was demonstrated that different edible mushroom species produce various organic acids from their fruiting bodies and mycelia [8]. Interestingly, organic acids may protect against various diseases because of their antioxidant activity in humans [8]. However, few studies have evaluated the antimicrobial potential against plant pathogenic bacteria using organic acids produced in the mycelial culture filtrate of mushroom. In this study, we characterized antibacterial substances against phytopathogenic bacteria from the culture products of L.
edodes as organic acids, focusing on oxalic acid, and evaluated
the biological activity of oxalic acid.

**Identification of antibacterial substances.** *Lentinula edodes* was cultured in 500 mL of sterile potato dextrose broth at 25°C for 28 days with standing. As shown in Fig. 1, the culture filtrate exhibited antibacterial activity, forming a clear zone of 22 mm on the paper disk method [9] against *Ralstonia solanacearum*, a causal agent of tomato bacterial wilt disease. To purify the antibacterial compound, the culture filtrate was subjected to octadecyl-silica column chromatography and eluted with different concentrations from 5% to 100% methanol. None of the 50-µL aliquots of methanol eluate showed an antimicrobial effect on the bacteria, but the water fraction that passed through the column maintained antibacterial activity. The pH of the passed fraction was 3.9, suggesting the presence of an organic acid. Therefore, qualitative analyses of organic acids from the culture filtrate of *L. edodes* were carried out by high-performance liquid chromatography (HPLC). HPLC was performed on a HITACHI Chromaster apparatus (Tokyo, Japan), which consisted of a pump, autosampler, column oven, UV-vis detector, and HPX-87 column (i.d. 4.6 × 300 mm, particle size of 5 µm, Aminex Therapeutics, Kenmore, WA, USA); temperature was maintained at 25°C. The flow rate was 0.6 mL/min. The mobile phase was 4 mM H₂SO₄ in water for a total running time of 25 min. The sample injection volume was 10 µL and the detection wavelength was 215 nm. Qualitative analysis of organic acids in the culture filtrate of *L. edodes* was performed by HPLC and the results were compared with those of authentic organic acids. Nine organic acids were detected and identified, as shown in Fig. 2. Oxalic acid was a dominant component in the culture filtrate of *L. edodes*, accounting for 50% of the HPLC analysis data. In addition to oxalic acid, phytic acid, malonic acid, and fumaric acid were major components.

**Quantitative analysis of oxalic acid.** Quantitative analysis of oxalic acid in the culture filtrate of *L. edodes* was carried out under the same HPLC conditions as were used in qualitative analyses. For quantitative analysis, a standard curve of oxalic acid was prepared in the concentration range of 200~2,000 µg/mL using four different concentrations. A

![Fig. 1. Antibacterial activity of culture filtrate of *Lentinula edodes* against *Ralstonia solanacearum*. 1, culture filtrate; 2, extract from fruiting body; 3, water; 4, tetracycline (30 mg/L).](image1)

![Fig. 2. Qualitative analysis of organic acids in the culture filtrate of *Lentinula edodes*. High-performance liquid chromatography analysis was performed on an Aminex HPX-87 column (i.d. 4.6 × 300 mm, particle size of 5 µm) at a flow rate of 0.6 mL/min. The mobile phase was 4 mM H₂SO₄ in water for a total running time of 25 min. The sample injection volume was 10 µL and detection wavelength was 215 nm.](image2)
line was plotted using linear regression of the peak area vs. concentration. The coefficient of correlation ($r^2$) was used to judge linearity. The coefficient of the correlation value for the standard curve of oxalic acid was 0.9982. HPLC analysis revealed that the concentration of oxalic acid was 708.711 ppm. Thus, the content of oxalic acid in the culture filtrate of *L. edodes* was 0.07%.

**Antibacterial activity of oxalic acid.** Commercial organic acid (Sigma, St. Louis, MO, USA) was tested for its antibacterial activity against *Ralstonia solanacearum* using the paper disk method [9]. Different oxalic acid concentrations (100 to 5,000 mg/L) were added to the filter paper disks (5 mm diameter), placed on nutrient agar, and overlaid with soft agar (0.8%) mixed with bacterial cells ($5 \times 10^6$). The plates were incubated at 28°C for 3 days. Clear zones that had formed around the paper disks were evaluated as growth inhibition of the bacterial cells. The antibacterial activities of nine different organic acids present in the culture filtrate of *L. edodes* shown in Fig. 2 were examined.

**Table 1.** Antibacterial activity of different oxalic acid concentrations against different plant pathogenic bacteria

| Bacterial strains                  | Initial viable count (CFU/mL) | Oxalic acids (mg/L) |
|-----------------------------------|------------------------------|--------------------|
|                                   |                             | 300    | 250    | 200    | 100    | 0      |
| *Ralstonia solanacearum*          |                              | 2.8 x 10^5 | 0 (0%) | 0 (0%) | 1.8 x 10^6 ± 0.06c | 9.7 x 10^5 ± 0.06b | 8.1 x 10^5 ± 0.06bc |
| *Pectobacterium carotovorum* subsp. *carotovorum* |                              | 1.2 x 10^5 | 0 (0%) | 0 (0%) | 1.6 x 10^5 ± 0.49bc | 2.1 x 10^5 ± 0.49ab |
| *Xanthomonas campestris* pv. *campestris* |                              | 3.6 x 10^4 | 0 (0%) | 4.0 x 10^5 ± 0.81c | 7.0 x 10^5 ± 0.23bc | 8.0 x 10^5 ± 0.82c | 1.6 x 10^5 ± 0.82a |
| *Pseudomonas tollassii* KACC10365 |                              | 1.7 x 10^4 | 1.4 x 10^5 ± 0.81b | 4.5 x 10^5 ± 0.12bc | 1.4 x 10^5 ± 0.49bc | 3.4 x 10^5 ± 0.49c | 4.8 x 10^5 ± 0.49ab |
| *Escherichia coli*, DH5α          |                              | 1.1 x 10^5 | 0 (0%) | 8.0 x 10^5 ± 0.72a | 8.0 x 10^5 ± 0.32a | 8.1 x 10^5 ± 0.10a | 8.3 x 10^5 ± 0.10a |
| *Agrobacterium tumefaciens*        |                              | 1.1 x 10^5 | 0 (0%) | 0 (0%) | 6.3 x 10^5 ± 0.19ab | 4.0 x 10^5 ± 0.39b | 1.3 x 10^5 ± 0.39ab |
| *Xanthomonas axonopodis* pv. *glycines* KACC1114 | | 5.0 x 10^4 | 0 (0%) | 0 (0%) | 6.7 x 10^5 ± 0.19a | 5.7 x 10^5 ± 0.17c |
| *Xanthomonas axonopodis* pv. *citri* KACC10443 | | 1.2 x 10^5 | 0 (0%) | 0 (0%) | 5.7 x 10^5 ± 0.14b | 4.7 x 10^5 ± 0.11c |
| *Xanthomonas campestris* pv. *glycines* KACC10491 | | 1.6 x 10^4 | 0 (0%) | 0 (0%) | 6.6 x 10^5 ± 0.21a | 3.9 x 10^5 ± 0.30b |
| *Xanthomonas axonopodis* pv. *vesicatoria* KACC12870 | | 4.2 x 10^4 | 0 (0%) | 0 (0%) | 3.5 x 10^5 ± 0.80bc | 1.3 x 10^5 ± 0.64a |
| *Xanthomonas oryzae* pv. *oryzae* KACC10859 | | 3.6 x 10^4 | 0 (0%) | 5.0 x 10^5 ± 0.50c | 6.3 x 10^5 ± 0.12c | 2.3 x 10^5 ± 0.22b |
| *Bacillus subtilis*               |                              | 3.2 x 10^5 | 1.2 x 10^6 ± 0.11a | 1.5 x 10^6 ± 0.32a | 4.8 x 10^5 ± 0.70a | 6.6 x 10^5 ± 0.23ab | 5.8 x 10^5 ± 0.33a |

The different letters are significantly ($p < 0.05$) different according to Duncan’s multiple test.
against *R. solanacearum*. Oxalic acid showed the highest antibacterial activity against *R. solanacearum*. The results are shown in Fig. 3. Each clear zone of 34, 27, and 12 mm was formed on concentrations of 5,000, 100, and 500 mg/L, but not on other concentrations of oxalic acid. Antibacterial activity on 500 mg/L oxalic acid was compared to the positive control, tetracycline (30 mg/L). Furthermore, the antibacterial effect of oxalic acid was evaluated against eight different phytopathogenic bacteria: *Xanthomonas campestris* pv. *campestris*, *R. solanacearum*, *Agrobacterium tumefaciens*, *Pectobacterium carotovorum* subsp. *carotovorum*, *X. oryzae* pv. *oryzae*, *X. axonopodis* pv. *citri*, *Pseudomonas tolaasi*, *X. axonopodis* pv. *glycines*, *X. axonopodis* pv. *vesicatoria*, *Escherichia coli*, and a gram-positive bacteria strain, *Bacillus subtilis*. Different concentrations of oxalic acid from 300 to 100 mg/L were added to nutrient broth containing different bacterial cells and cultured at 28°C for 24 hr. The antibacterial activity was measured as the number of living cells. As shown in Table 1, oxalic acid exhibited antibacterial activities against the bacterial strains tested at the minimum inhibitory concentration (MIC) of 250 mg/L, except *X. campestris* pv. *campestris*, *A. tumefaciens*, *P. tolaasi*, *E. coli*, and *Bacillus subtilis*. *Pectobacterium carotovorum* subsp. *carotovorum*, *X. axonopodis* pv. *citri*, *X. axonopodis* pv. *glycines*, and *X. axonopodis* pv. *vesicatoria* were the most sensitive, and cell growth was inhibited at an MIC of 200 mg/L. Interestingly, oxalic acid, like antibiotics such as tetracycline, had a bactericidal effect and the growth of bacterial cells could not be recovered on nutrient media. In preliminary experiments, the pH of media containing oxalic acid was pH 3.5–4.5; we further evaluated whether the antibacterial activity of oxalic acid was a result of low pH. The pH 3.5–4.5 media were adjusted with HCl and the *R. solanacearum* cells were cultured in the media. The bacterium was grown in the media, although the growth rate was slightly different corresponding to pH (data not shown), indicating that low pH is not main factor affecting antibacterial activation. Taken together, the results suggest that oxalic acid is critical factor responsible for antibacterial activity.

Oxalic acid, a saturated short chain dicarboxylic acid, occurs naturally in many plants [10, 11] and fungi [12] and forms insoluble calcium or magnesium oxalate crystals, soluble sodium, or potassium oxalate [13]. In previous study, different organic acids were identified in Japanese apricot fruit by HPLC analysis and their antibacterial activities were determined against *E. coli*, *B. subtilis*, *Staphylococcus aureus*, and *Streptococcus suis* [11, 14]. However, oxalic acid showed relatively low antibacterial activities towards these bacterial species with an MIC of 1,300 mg/L compared to the MIC of 500 mg/L found in this study. Oxalic acid produced by plant pathogenic fungi provide a low pH, which results in maximal activities of fungal enzymes that degrade the cell wall of the host plant [15]. In artificial cultivation of *L. edodes* mushroom, *L. edodes* may degrade wood sawdust by producing lignocellulytic enzymes (laccase, cellulase, and xylanase) and the resulting product, glucose, is used in energy metabolism. Thus, oxalic acid creates low pH conditions to optimize the enzymatic activities.

Oxalic acid in the SMS of *L. edodes* was quantitatively analyzed. The SMS after 4 cycles of harvest was obtained.

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**Fig. 4.** High-performance liquid chromatography analysis of oxalic acid in water extract from spent mushroom substrate of *Lentinula edodes*.
from a mushroom farm, mixed with water (1:3 ratio w/v), and incubated with shaking for 2 hr. The mixture was then filtered through two layers of Miracloth (Calbiochem, La Jolla, CA, USA), centrifuged for 10 min at 5,000 ×g and the supernatant was used as the water extract of SMS. Water extract from the natural mushroom substrate that had not been inoculated with L. edodes was used as a control. To quantitatively analyze oxalic acid, both samples were subjected to HPLC. The results are shown in Fig. 4. The one peak at 6.63 min indicating oxalic acid was detected at 1000 mAU in the water extract of a SMS sample, but not in the water extract from natural mushroom substrate. *Lentinula edodes* produces oxalic acid as the main organic acid during mushroom cultivation. Thus, the extract of SMS of *L. edodes* may be an eco-friendly control agent for bacterial plant diseases.

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