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Growth Inhibition on the Strain Isolated from Spoiled Red Bean Paste

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Abstract Growth inhibitory effects of ethanol extracts of green tea and pine needles on *Bacillus stearothermophilus* isolated from spoiled red bean paste were detected at concentrations higher than 750 ppm, and antimicrobial activity of pine needle extract was slightly higher than that of green tea exract. Growth inhibitory effect of pine needle extract in nutrient broth adjusted to pH 6.0, water-activity 0.92, and 45 °Brix was observed at 500 ppm. These results indicated growth of *B. stearothermophilus* could be inhibited by adding pine needle and green tea extracts.

Key words: *Bacillus stearothermophilus*, red bean paste, growth inhibitory effect, pine needle extract, green tea extract

Introduction

Red bean paste, largely used as a filling material for bakery products, has high sugar content with 50°Brix, pH 6.48, and water-activity of 0.92 (1). However, it is susceptible to microorganism spoilage due to its low acidity and higher water-activity than that of the lowest water-activity of 0.91 the maximum activity allowable for the growth of most bacteria (2). In addition, due to its high viscosity, it has low heat penetration property and thus cannot undergo thermal sterilization, resulting in frequent spoilage during summer.

The use of various preserving methods including thermal sterilization has been considered to improve the preservation of food. In recent years, however, because consumers prefer natural preservatives to synthetic ones, intensive efforts have been made to identify antimicrobial substances contained in plants and animals (3-6).

Bacillus stearothermophilus (7) isolated and identified from spoiled red bean paste proliferates at high temperatures and produces high heat-resistant spores. It is also known as a saprogenous bacillus of bottled food and hot-packed food (8). Moreover, studies on the sterilization effects of ozone on *B. stearothermophilus* in various foods (9), and the inhibition effects of bacteriocins, which is produced by lactic acid bacteria (10), were also performed.

Green tea extract showed antimicrobial activity at concentrations of 0.7, 0.05-0.1, and 0.25-0.35%, respectively, against the spoiling microorganisms of boiled rice such as *Bacillus subtilis* (11), food-borne bacteria including *Staphylococcus aureus* (12), and spoiling microorganism of bread such as *B. subtilis* and *B. cereus* (13). In addition, several reports have been made on the growth inhibitory effects of the pine needle extract against *Listeria monocytogenes* (14) and *Vibrio* species (15), and of the pine needle essential oil for food-borne bacteria (16).

This study investigated the effects of the ethanol extracts of green tea and pine needle on the inhibition of *B. stearothermophilus* (7).

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Materials and Methods

Strains and medium Pure *B. stearothermophilus* (7) strain, isolated and identified from spoiled red bean paste products (5 kg; Daedoo Food Co., Ltd., Gunsan, Korea; Packed by Nylon & Polyethylene), was cultivated in a nutrient agar and nutrient broth (Oxoid Ltd., Basingstoke, Hampshire, England), freeze-dried, and stored in 50% glycerol stock in a -60°C refrigerator.

Preparation of the pine needle and green tea extract Pine needles collected from an autogenous pine tree (*Pinus densiflora* S. et Z.) growing on a hill around Chonbuk National University (Jeonju, Korea) in April, 2003 were washed, ground using a Waring blender (31BL91, New Hartford, CT, USA), blended (500 g pine needle powder/ 2.5 L of 75% ethanol), placed in a flask with a reflux condenser tube, and extracted in an 80°C water bath for 3 hr. The extract was then filtered using a Whatman No.2 filter paper (Whatman Int. Ltd., Maidstone, Kent, England), and the residuals were concentrated at 45°C using a vacuum evaporator (Eyela, Tokyo Rikakikai Co. Ltd., Tokyo, Japan). The content of solid matter for the liquid concentration where the agent was completely evaporated was measured based on the evaporation residues of the sample (1 g) after drying at 105°C. The pine needle extract was dissolved in 75% ethanol to examine its antibacterial activity.

Bosung leaf green tea (Bosung, Korea) purchased from a local market was extracted and its antibacterial activity tested by the same procedures applied to the extract of pine needles.

Measurement of antibacterial activity The ethanol extracts of green tea and pine needles were dissolved in ethanol, and the microorganisms were filtered out using a membrane filter (0.2 μm, Millipore Co., Bedford, MA, USA). Each extract (0.1 mL) was added to the nutrient broth (9.8 mL) to adjust the concentration to 250, 500, 750, 1000, and 2000 ppm solid matter. Bacterial culture

medium (0.1 mL, 10^5-10^6 CFU/mL) was also added to nutrient broth. O.D. was measured (600 nm) at 37°C using Bioscreen C for 72 hr at 12-hr intervals. The amount of ethanol as much as the use of ethanol to dissolve the sample was added to the comparative group in order to consider the antibacterial activity. For comparison of antibacterial activity, control group was added with equal amount of ethanol used to dissolve the extract samples.

Results and Discussion

Growth inhibitory effects by green tea extracts The growth inhibitory effects of the green tea ethanol extract on *B. stearothermophilus* are shown in Fig. 1. In 250 and 500 ppm ethanol extract-treated groups, very low O.D. was observed up to 24 hr, which then increased rapidly thereafter, and no significant differences were observed after 48 and 72 hr compared with the control group. However, the O.D. values showed almost no change from the initial stage for the 750, 1000, and 2000 ppm-treated groups, indicating that the growth was strongly inhibited by the treatment. Kim *et al.* (13) reported that the addition of green tea extract over 1% to the bread-spoiling bacteria, such as *B. subtilis*, *B. pulmilus*, and *B. cereus*, inhibited their growths. In addition, Roh *et al.* (11) reported that the addition of green tea extract to the cooked rice-spoiling bacteria *B. subtilis* at 500 and 1000 ppm resulted in antibacterial activity. Park (17) reported that (-) epicathechin gallate and (-)epigallocathechin gallate, flavonol derivatives of green tea, had stronger antimicrobial activities on the pathogenic bacteria than (-)epicathechin and (-) epigallocathechin. From these results, it can be verified that the growth of *B. stearothermophilus* isolated from spoiled red bean paste was strongly inhibited by the ethanol extract of green tea at over 750 ppm.

Growth inhibitory effects by pine needle extracts Growth of the isolated strain was not observed upon the application of the pine needle extract at over 750 ppm to the spoiling bacteria of red bean paste (Fig. 2). Lim *et al.* (14) reported that the strongest antimicrobial activity was shown with the treatment of 40 mg/mL pine needle ethanol extract to *L. monocytogenes*. Park and Lee (15) reported that the number of strains decreased by 2 log cycles with the application of 1% pine needle ethanol extract to *V. parahaemolyticus* and *V. vulnificus*. Jeon *et al.* (18) reported that (1*R*)-(+)-α-pinene, limonene, and terpinolene, constituents of *Pinus densiflora* leaves, strongly inhibited the growths of *Clostridium perfringens*, *S. aureus*, and *Escherichia coli*. Results of these reports suggest that the isolated strains show lower tolerance to the extract of pine needles than *L. monocytogenes* and *Vibrio* species.

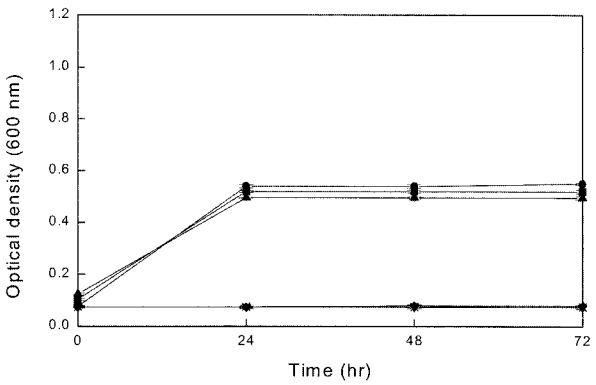

Fig. 2. Growth inhibitory effects of the ethanol extract of pine needles on *Bacillus stearothermophilus* isolated from spoiled red bean paste for 72 hr at 45°C. See footnote in Fig. 1.

Growth inhibitory effects by pine needle extracts in the conditions similar to red bean paste The growth inhibitory effects of pine needle extracts on *B. stearothermophilus* in the nutrient broth (pH 6.0, 0.92 water-activity adjusted with glycerol, and 45 °Brix adjusted with sugar) similar to the red bean paste are shown in Fig. 3.

The number of strains increased by 1-1.5 log cycle after 72 hr for the control group. The initial number of strains for the 100 ppm-treatment group was maintained up to 72 hr. The number of strains for the 250 ppm-treatment group decreased by 2-3 log cycles up to 48 hr then increased

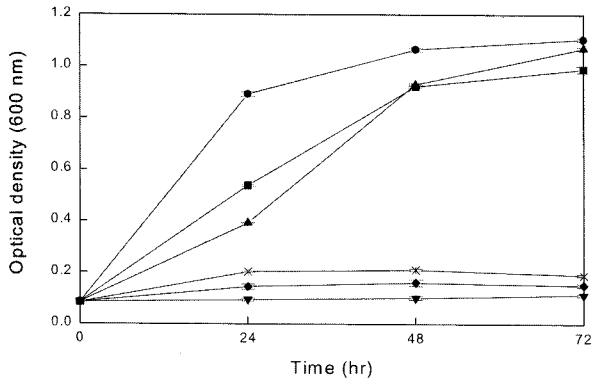

Fig. 1. Growth inhibitory effects of the ethanol extract of green tea on *Bacillus stearothermophilus* isolated from spoiled red bean paste for 72 hr at 45°C. - ● -: control, -■-: 250 ppm, -▲-: 500 ppm, -x-: 750 ppm, -◆-: 1000 ppm, -▼-: 2000 ppm. Vertical bars represent standard deviation (n=3).

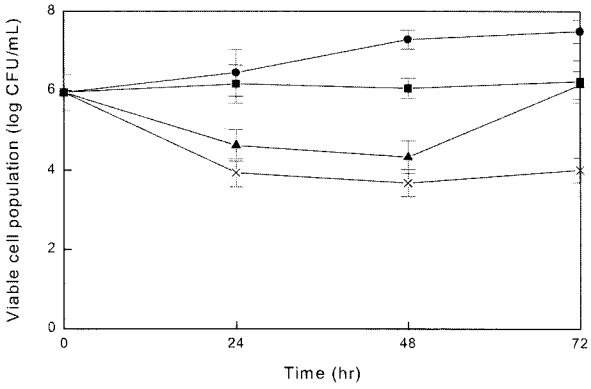

Fig. 3. Changes of total viable cell population of *Bacillus stearothermophilus* isolated from spoiled red bean paste with various concentrations of pine needle extracts in adjusted medium (pH 6.0, Aw 0.92, 45 °Brix) for 72 hr at 45°C. -●- : control, -■ -: 100 ppm, -▲ -: 250 ppm, -x-: 500 ppm. Vertical bars represent standard deviation (n=3).

thereafter. Furthermore, the growth of the isolated strains was inhibited, showing a decrease of 2-3.5 log cycles compared with the control group 24 hr after treatment of 500 ppm pine needle extract.

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