Evaluation of Antagonistic Activities of *Bacillus subtilis* and *Bacillus licheniformis* Against Wood-Staining Fungi: *In Vitro* and *In Vivo* Experiments

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(Received January 12, 2009 / Accepted April 14, 2009)

The antifungal activity of bacterial strains *Bacillus subtilis* EF 617317 and *B. licheniformis* EF 617325 was demonstrated against sapstaining fungal cultures *Ophiostoma flexuosum*, *O. tetropsis*, *O. polonicum*, and *O. ips* in both *in vitro* and *in vivo* conditions. The crude active supernatant fractions of 7 days old *B. subtilis* and *B. licheniformis* cultures inhibited the growth of sapstaining fungi in laboratory experiments. Thermostability and pH stability of crude supernatants were determined by series of experiments. FT-IR analysis was performed to confirm the surface structural groups of lipoproteins present in the crude active supernatant. Partial purification of lipopeptides present in the crude supernatant was done by using Cellulose anion exchange chromatography and followed by Sephadex gel filtration chromatography. Partially purified compounds significantly inhibited the sapstaining fungal growth by *in vitro* analysis. The lipopeptides responsible for antifungal activity were identified by electrospray ionization mass spectrometry after partial purification by ion exchange and gel filtration chromatography. Four major ion peaks were identified as m/z 1023, 1038, 1060, and 1081 in *B. licheniformis* and 3 major ion peaks were identified as m/z 1036, 1058, and 1090 in *B. subtilis*. In conclusion, the partially purified lipopeptides may belong to surfactin and iturin family. *In vivo* analysis for antifungal activity of lipopeptides on wood was conducted in laboratory. In addition, the potential of extracts for fungal inhibition on surface and internal part of wood samples were analyzed by scanning electron microscopy.

**Keywords**: *Bacillus subtilis*, *Bacillus licheniformis*, lipopeptides, surfactin, iturin, *Ophiostoma* sp.

A serious damage in natural color of wood is caused by sapstaining fungi which create a significant economic loss in wood industries worldwide; however the strength of wood is not affected by the sapstaining fungus. Staining in wood by fungus is created due to the production of melanin in ray parenchyma tissues and cell lumens of fungal hyphae. *Ophiostoma* sp. is one of the major genera of sapstaining fungi. Many reports were published concerning the staining on wood, and several preservatives and remedies are recommended for inhibiting sapstaining fungal growth on wood. Chemical preservatives are predominantly used in wood industries to control sapstaining fungal growth. However, these preservatives are extremely toxic to the environment and human health. Thus, many researches had been focused on the development of natural resources and biological compounds as potential preservatives. The biological control of sapstaining fungal growth is of tremendous economic significance in the wood industries. Many authors reported the protection of wood by biological control (Barr, 1975; Bernal et al., 2002; Feio et al., 2004). Some researchers were already reported the biological control of wood staining fungi by albinio wood stain fungi and bacteria (Payne et al., 2000; Held et al., 2003; Cho et al., 2008)

Biological control agents are widely used in many fields, including agriculture, medicine, and forestry products. *Bacillus* sp., is a broadly used genera in aspects of biological control in most of the fields. *Bacillus* sp. has received greater attention because of their potential to produce wide range of secondary metabolites with strong antimicrobial and antifungal activity and consequently used in industrial scale. Those metabolites are thermostable in nature and active in both alkaline and acidic conditions. Most of the metabolites produced by *Bacillus* sp. having less than 2000 Da molecular weight and sharing general amino acid sequence composed of 7 amino acids and generally has β-amino fatty acid in the peptide linkage (Haavik and Thomassen, 1973; Vanittanakom et al., 1989). These lipopeptides are synthesized by large multi enzyme complexes instead of ribosome synthesis manner. The non-ribosme synthesized lipoproteins are mainly belongs to iturin, surfactin, fengycin, pilipastatin, the di- and tripeptides such as bacilysin and the phosphono-oligopeptide and rhizoctin group (Moyne et al., 2001). The lipopeptides produced by *Bacillus* sp. are mainly classified into three families, such as iturin, surfactin, and fengycin. All three peptide groups are exhibiting greater antifungal activity (Moyne et al., 2001; Feio et al., 2004).
Many authors have already been reported the isolation, purification, and characterization of lipopeptides against the plant pathogenic fungal groups (Brown and Bruce, 1999; Moyne et al., 2001; Feio et al., 2004; Melent’ev et al., 2006). The structures of lipopeptides were determined and the possible mechanism between the antagonistic activity of lipopeptides and fungal samples were also discussed earlier (Melent’ev et al., 2006; Romero et al., 2007; Volpon et al., 2007). However, evidence for antimicrobial and antifungal activity of *Bacillus* sp. is not well discussed in *in vivo* conditions. Although, to our knowledge, this is the first approach to identify the antifungal compounds from *Bacillus subtilis* and *Bacillus licheniformis* particularly active against sapstaining fungal growth. The present work was carried out to isolate, identify, purify, and characterize the compounds from *Bacillus subtilis* and *Bacillus licheniformis* with strong antifungal activity and the potential antifungal activity of purified compounds were determined in both *in vitro* and *in vivo* conditions.

**Materials and Methods**

**Microorganisms**

**Fungal samples**

The following sapstaining fungal samples were used in the present study: *Ophiostoma flexuosum* (363175), *Ophiostoma tetrupii* (363182), *Ophiostoma polonicum* (343181), and *Ophiostoma ips* (363176). All the fungal samples were obtained from CABl, Bioscience, UK Centre, formerly called as International Mycological Institute (IMI). The fungal cultures were grown on 2% MEA (Becton, Dickinson and Company, USA) medium as pre-inoculums at 25°C for 4–7 days.

**Bacterial samples**

*Bacillus subtilis*, strain EF 617317 and *B. licheniformis*, strain EF 617325 isolated from the Chonbuk National University agricultural field soil were obtained from Biocontrol laboratory, Institute of Agriculture and Life Sciences, Chonbuk National University, Republic of Korea. Cultures were grown in LB (Becton, Dickinson and Company) agar plates.

**Antagonistic activity assay**

Antagonistic activity of *B. subtilis* EF 617317 and *B. licheniformis* EF 617325 was determined by petri-plate assay method. Actively growing mycelial edges of fungal samples *O. polonicum*, *O. flexuosum*, *O. tetrupii*, and *O. ips* were placed on the center of petri-plate containing LB agar, individually and bacterial cultures of *B. subtilis* strain EF 617317 and *B. licheniformis* strain EF 617325 were inoculated on both corners of each fungal samples *O. polonicum*, *O. flexuosum*, *O. tetrupii*, *O. ips*, individually. Control plates were maintained only with fungal samples without inoculation of bacterial samples. Plates were incubated at 25°C for 7 days. The growth inhibition of fungal hyphae of all samples was observed visually and antagonistic activity of the bacterial samples was confirmed by the assay results.

**Preparation of antagonistic compounds from bacterial cultures**

The bacterial isolates *B. subtilis* and *B. licheniformis* were grown in 100 ml of LB broth containing 0.5 g yeast extract, 1.0 g peptone and 1.0 g NaCl, pH 7.2 at 25°C, 200 rpm for 7 days. The cultures were centrifuged at 10,000 rpm for 20 min at 4°C after 7 days of incubation. The supernatants of both *B. subtilis* and *B. licheniformis* cultures were separately collected and pellets were discarded. The active supernatants were acidified to pH 2.0 with 2 M HCl. The acidified supernatants were centrifuged at 10,000 rpm for 30 min at 4°C, supernatants were discarded after centrifugation. Both pellets were washed twice separately with 40% methanol. Completely washed pellets were separated into three parts. FT-IR analysis was carried out with one part of the completely dried pellets, another part of the dried pellets were dissolved in 40% methanol to determine the antifungal activity against wood staining fungi and final part of the pellets were separately dissolved in 50 mM Tris-HCl, pH 7.5 buffer for partial purification and identification of active compounds.

**Determination of antifungal activity of cell free fractions**

Antifungal activities of cell free fractions (dissolved in 40% methanol) were detected by disc diffusion method in 2% MEA agar plates. Whatman No. 1 filter paper was used to prepare the discs (approximately 6 mm in diameter). Disks were sterilized at 121°C for 20 min. Twenty microliters of the active 40% methanol dissolved fractions of both *B. subtilis* and *B. licheniformis* were added drop by drop in sterilized disks, then allowed for dry in laminar air flow chamber. Activated discs of both *B. subtilis* and *B. licheniformis* were placed on both sites of fungal samples *O. polonicum*, *O. flexuosum*, *O. tetrupii*, and *O. ips*. Control plates were maintained separately, Whatman filter discs used in control plates were dipped with 40% methanol. All plates were incubated at 25°C for 12 days.

**FT-IR analysis of active supernatants**

The active pellets of both *B. subtilis* and *B. licheniformis* were completely dried at 37°C, separately. A small amount of dried pellets were mixed with appropriate amount of potassium bromide, individually. The mixtures were thoroughly ground in a sterilized mortar and pestle, and then subjected to high pressure (18 psi) to form a small pellets about 1 mm in thick and 1 cm in diameter. The resulting pellets were transparent and were used to test the surface functional groups by Fourier Transform Infrared Spectroscopy (Spectrum GX, USA), pellets were scanned from 4000 to 400 cm⁻¹ at a resolution of 4 cm⁻¹.

**Partial purification of active compounds**

The partial purification and identification of active compounds responsible for antifungal activity was carried out by modified method of Zhang et al. (2007). Two Cellulose DE52 (Whatman Cellulose) columns were separately prepared for the first purification of active supernatant fractions of both *B. subtilis* and *B. licheniformis*, dissolved in 50 mM Tris-HCl (pH 7.5) buffer. Both columns were individually equilibrated with 50 mM Tris-HCl (pH 7.5) buffer containing 50 mM NaCl. The first wash of both columns were made by 150 ml of Tris-HCl (pH 7.5) and the second wash was made by 150 ml of linear NaCl gradient with the...