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

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The antifungal activity of bacterial strains *Bacillus subtilis* EF 617317 and *B. licheniformis* EF 617325 was demonstrated against sapstaining fungal cultures *Ophiostoma flexuosum*, *O. tetropii*, *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 albino 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-ribosome synthesized lipoproteins are mainly belongs to iturin, surfactin, fengycin, plipastatin, the di- and tripeptides such as bacilysin and the phosphono-oligopeptide and rhizocticin groups (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).

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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* condition. 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 tetropii* (363182), *Ophiostoma polonicum* (343181), and *Ophiostoma ips* (363176). All the fungal samples were obtained from CABI, 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. lichneiformis*, 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. lichneiformis* EF 617325 was determined by petri-plate assay method. Actively growing mycelial edges of fungal samples *O. polonicum*, *O. flexuosum*, *O. tetropii*, 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. lichneiformis* strain EF 617325 were inoculated on both corners of each fungal samples *O. polonicum*, *O. flexuosum*, *O. tetropii*, *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. lichneiformis* 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. lichneiformis* 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). Discs were sterilized at 121°C for 20 min. Twenty microliters of the active 40% methanol dissolved fractions of both *B. subtilis* and *B. lichneiformis* were added drop by drop in sterilized discs, then allowed for dry in laminar air flow chamber. Activated discs of both *B. subtilis* and *B. lichneiformis* were placed on both sites of fungal samples *O. polonicum*, *O. flexuosum*, *O. tetropii*, 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. lichneiformis* 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

concentration from 50 mM to 1,000 mM NaCl at flow rate of 1 ml/min. The final wash was made by 50 mM Tris-HCl (pH 7.5) at flow rate of 1 ml/min. All fractions were observed at 280 nm. The second purification of antifungal fractions was made by Sephadex G-50 (Sigma-Aldrich). Two Sephadex G-50 columns were separately packed for purification of antifungal fractions of *B. subtilis* and *B. licheniformis*. Both columns were equilibrated with 100 ml of distilled water and then the elution was made by distilled water at flow rate of 1 ml/min. Collected fractions were observed at 280 nm. The major peaks were tested for antifungal activity against wood staining fungi in *in vitro* condition. Five hundred microliter of the major peaks were spread over 2% MEA plates, step by step and then allowed for air dry in laminar air flow chamber. One hundred microliter of spore suspension of each sapstaining fungi were spread over the antifungal fractions in separate plates. While 500 μl of 50 mM Tris-HCl, pH 7.5 was spread on the control plates instead of active fractions. The plates were incubated at 25°C for 2 days. Antifungal activity of the compounds was determined based on visual screening of growth inhibition zone of fungal spores. Identification of active compounds was done by Mass Spectrometry. The molecular weights of active compounds were determined by electrospray ionization mass spectrometry (Agilent 1100 series LC-MSD Trap 00099). The mass spectrometry operated with 19 psi pressure and dry temperature at 320°C.

Thermostability and pH stability of active fractions

The active supernatant fractions (crude extracts) of 7 days old cultures of *B. subtilis* and *B. lichneiformis* were used for thermal stability tests. The active supernatants were maintained in water bath at 20, 37, 50, 70, and 100°C for 30 min and then antifungal assay was carried out against fungal samples *O. polonicum*, *O. flexuosum*, *O. tetropii*, *O. ips*. The active supernatant fractions were adjusted to pH 2, 2.5, 3, 4, 5, 6, 7, 8, 9, and 10 at 25°C for 24 h. Different pH fractions were tested against all 4 fungal samples to demonstrate their antifungal activity. Disc diffusion method was carried out to demonstrate the thermo stable property and pH stability of active fractions.

In vivo analysis of activity of bacterial active supernatants on wood samples

Antifungal activity of active supernatant fractions on wood: To test the antagonistic activity of active supernatant fractions against wood staining fungi *in vivo*, an additional incubation method was performed. *Pinus densiflora* and *Pinus radiata* log sections and pickle-jars were used for this experiment. Uncontaminated wood logs were purchased from Gunsan City Wood Industry, Republic of Korea. The wood blocks were selected randomly and those samples were placed in a plastic bag, properly labeled, and then taken to the laboratory. Wood specimens were prepared from *Pinus densiflora* and *Pinus radiata* log sections in Chonbuk National University Wood Center (60 mm × 40 mm × 10 mm in size). Wood specimens were sterilized at 121°C for 20 min. Sixty ml of 2% MEA medium was poured into each pickle-jar individually and 4 fungal samples were inoculated in pickle-jar, separately then kept in incubator at 20°C for 6~7 days until the fungal hypha reaches the edge of the jar. After complete growth of fungi on surface of the medium, two wood samples (*Pinus densiflora* and *Pinus radiata*) were placed on each jar. These jars were considered as the control. Eight replicates were maintained for each fungal sample. Wood specimens of *P. densiflora* and *P. radiata* were kept in cell free crude active supernatant fractions of *B. subtilis* and *B. lichneiformis* for 15 min, separately and then air dried in laminar air flow chamber. These treated wood specimens were placed on the surface of each fungus grown on 2% MEA in the pickle jar. One treated specimen from *P. densiflora* and one from *P. radiata* were added to each pickle-jar. Four replicates were maintained for each test. Pickle-jars were sealed with parafilm tape and incubated in humidity chamber (moisture content around 120%) at 25°C for 16 weeks.

Table 1. Antifungal activity of *B. subtilis* EF 617317 and *B. licheniformis* EF 617325 on LB agar. Values indicating the distance (mm) between the fungal mycelial edge and border of bacterial colony

Fungal samples	*B. subtilis* (mm)	*B. licheniformis* (mm)
O. polonicum	7,8	5,8
O. ips	4,6	5,10
O. tetropii	6,4	4,6
O. flexuosum	4,6	5,8

Analysis of fungal growth on wood samples

After 16 weeks of incubation of wood samples with sapstaining fungi, the samples were removed from the pickle-jar and stain category number has given to all wood samples depending on the severity of fungal staining on wood samples (Table 2). The visual data analysis was carried out based on the method of Thwaites *et al.* (2004). Statistical analysis of

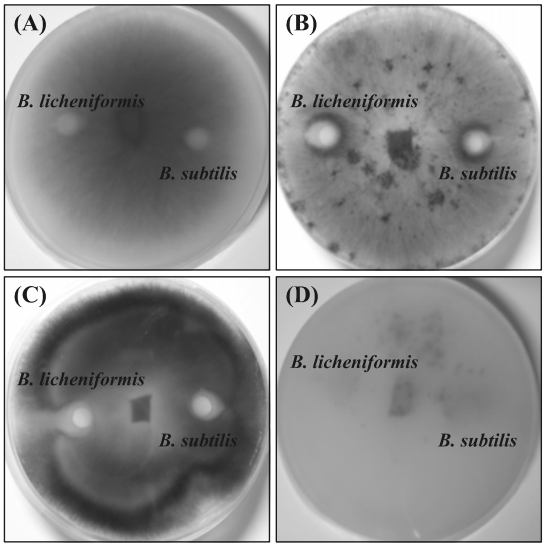

Fig. 1. Photographs of the antifungal test results of the cell free supernatants of *B. licheniformis* EF 617325 and *B. subtilis* EF 617317: *O. ips* (A), *O. polonicum* (B), *O. flexuosum* (C), *O. tetropii* (D).

Table 2. Visual fungal staining category numbers and detailed descriptions, used to determine the sapstain coverage on wood in *in-vivo* experiment (based on Thwaites *et al*., 2004)

Category of staining degree or severity	Description
0	No stain (0%)
1	Minimal stain (1~10%)
2	Mild stain (11~20%)
3	Moderate stain (21~50%)
4	Heavy stain (51~80%)
5	Severe stain (81~100%)

visual stain data was performed using the program SigmaPlot 10.0. Fungal colonization on wood samples and hypha penetration into wood samples were analyzed by Scanning Electron Microscopy. SEM investigation was mainly used to study about the inhibition capacity of extracts in internal wood decay. Samples were obtained for SEM analysis by pouring the liquid nitrogen on wood samples, after complete evaporation of liquid nitrogen the wood samples were sectioned by sterilized knife to make small pieces then the samples were dehydrated through an ethyl alcohol series (20%, 30%, 40%, 50%, 60%, and 70%, each 10 min.). After dehydration, samples were allowed for air-drying and were coated with gold by ion sputtering (JEOL JFC-1200 fine coater) and observed under SEM (JSM-5200).

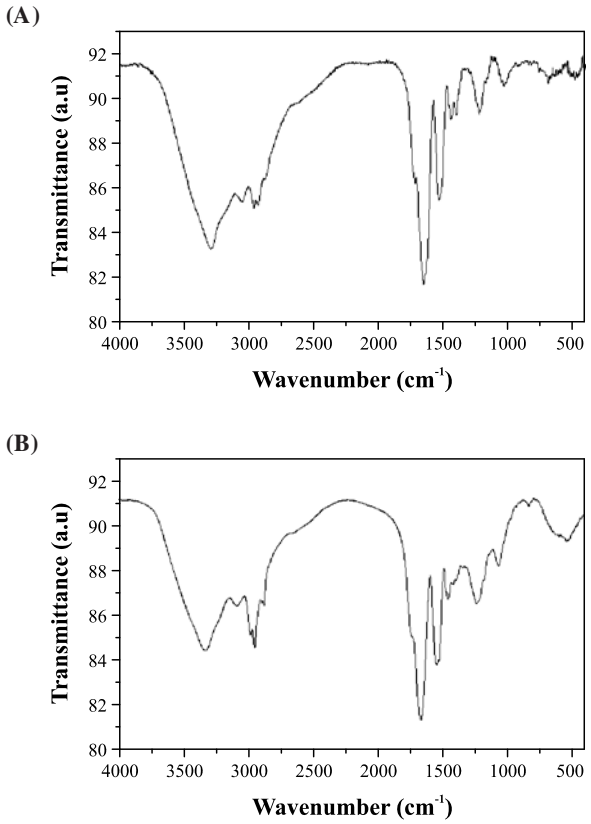

Fig. 2. FT-IR spectrum of active supernatant fractions of *B. licheniformis* EF 617325 (A) and *B. subtilis* EF 617317 (B).

Results

Antifungal activity of *B. subtilis* and *B. licheniformis*

Both the bacterial strains, *B. subtilis* EF 617317 and *B. licheniformis* EF 617325 strongly inhibited the mycelial growth of *O. polonicum*, *O. flexuosum*, *O. tetropii*, and *O. ips* on LB plates. The inhibition rate was indiciated by inhibition zone. The details of zone of mycelial growth inhibition range were given in Table 1.

Antagonistic activity of cell free bacterial fractions

Antifungal activities of cell free factions were determined by disc diffusion method under the laboratory condition (Fig. 1). The mycelial growth of sapstaining fungal samples, *O. polonicum*, *O. flexuosum*, *O. tetropii*, and *O. ips*, were inhibited by cell free fractions of both *B. subtilis* and *B. licheniformis*. A clear zone of inhibition was observed around all the activated discs. *O. flexuosum* was more sensitive towards the bacterial cell free fractions (Fig. 1C). Thus, the experiment supports the antifungal activity of partially purified compounds of both *B. subtilis* and *B. licheniformis*.

Identification of partially purified fractions

The surface structure and active groups of active compounds in active supernatant was determined by FT-IR. Figure 2 displays the FT-IR spectra of active supernatant fraction. The surface groups of active supernatant compounds were described as follows: The groups were observed between

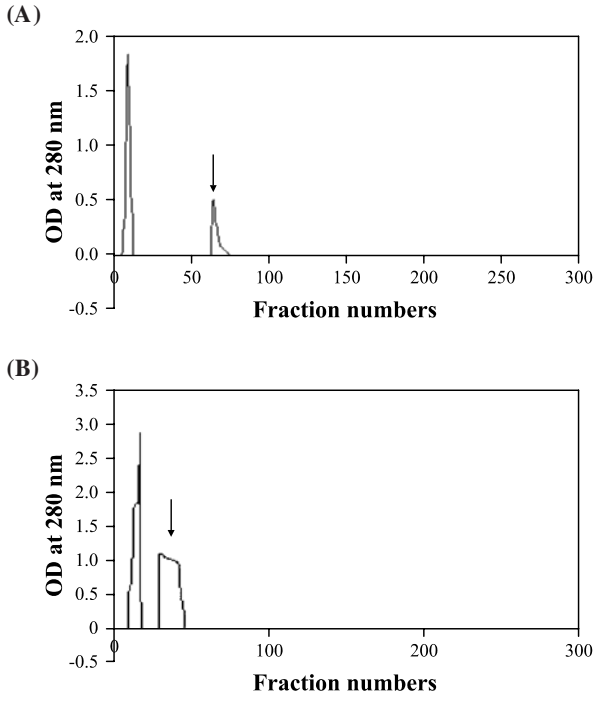

Fig. 3. Anion exchange chromatography purification of active compounds on cellulose DE 52 Column. Column was eluted with Tris-HCl buffer at flow rate of 1 ml/min. and fractions were detected at 280 nm, *B. licheniformis* EF 617325 (A); *B. subtilis* EF 617317 (B).

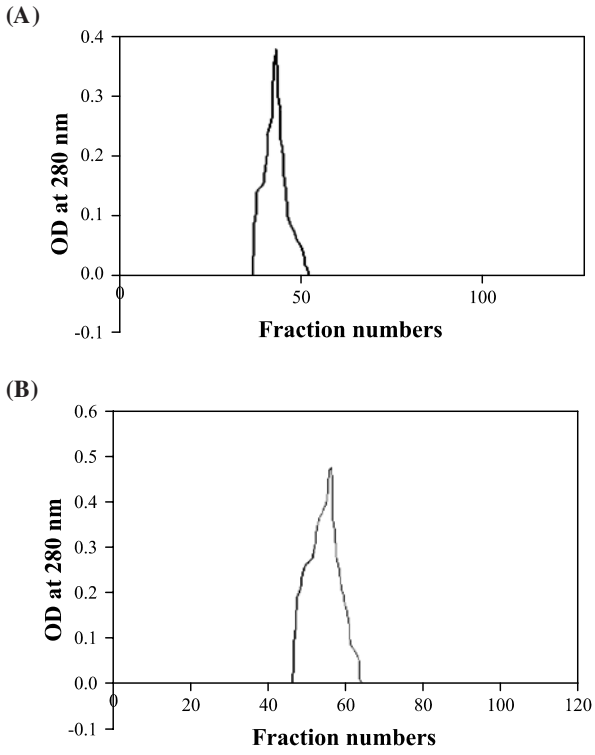

Fig. 4. Sephadex gel filteration chromatography purification of active compounds Column was eluted with distilled water at flow rate of 1 ml/min. and fractions were detected at 280 nm, *B. licheniformis* EF 617325 (A); *B. subtilis* EF 617317 (B).

3295 to 2929 cm^{-1} in *B. licheniformis* (Fig. 2A) and 3302 to 2856 cm^{-1} and 1655 to 1535 cm^{-1} in *B. subtilis* (Fig. 2B). The broad bands observed around 3300 cm^{-1} are attributed for NH groups and bands appeared at 1655 and 1535 cm^{-1} reflected the amide I and amide II bands, respectively. Partial purification of active compounds dissolved in 50 mM Tris was achieved by using anion exchange chromatography and followed by Sephadex gel filtration chromatography. After first purification by cellulose column, all fractions were observed at 280 nm and the peaks appeared at retention time 63 to 69 in *B. licheniformis* (Fig. 3A) and 29 to 40 in *B. subtilis* (Fig. 3B) were separately collected and allowed for the second purification by Sephadex gel purification. The peaks appeared in gel filtration chromatography for both *B. licheniformis* (Fig. 4A) and *B. subtilis* (Fig. 4B) showed significant antifungal activity by *in vivo* assay method. Those peaks were separately collected and utilized for compound identification by mass spectrometry. The molecular masses of active fractions of both *B. subtilis* and *B. licheniformis* were confirmed by MS spectrometry. Four major ion peaks were identified at *m*/*z* 1023, 1038, 1066, and 1081 in *B. licheniformis* (Fig. 5A) and three major ion peaks were observed at *m*/*z* 1036, 1058, and 1090 in *B. subtilis* (Fig. 5B). These ion peaks were may differ depends on the length of their fatty acid chains.

Thermostable character and pH stability of active fractions

Thermostability and pH stable characters of both bacterial active supernatant fractions were confirmed by series of experiments. The initial activities of both fractions were maintained throughout the series of temperatures 20, 37, 50, 70, and 100°C for 30 min. The similar active result was also observed in pH stability test. No significant activity loss has been observed between pH 2 to10 at 25°C for 24 h.

Antagonistic activity of active supernatants in *in vivo* condition

Fungal growth and staining on treated and control wood samples were visually observed after 16 weeks of incubation. Ranking was provided to all samples based on the severity of fungal staining (Table 2). The fungal staining was ranked from minimal (1∼10%) to severe staining (81∼100%). No significant differences between replicates. The results of *in vivo* experiments were provided in Table 3. Fungal staining reduction was observed on treated wood samples. A significant fungal staining reduction was observed on treated *Pinus densiflora* wood samples. This may be due to the naturally amended polyphenolic groups on *Pinus densiflora*. *In vivo* experiments, *O. polonicum* and *O. ips* were more sensitive to biological agents than *O. tetropii* and *O. flexuosum* (Table 3).

The SEM studies provided information on fungal growth inhibition on wood surface and cell wall degradation (Fig. 6), especially on inner part of wood cells. Amended fungal growth on the surface of wood samples was observed in control blocks (Fig. 6B). The hyphae penetrating through the inner surface of the cell to cell passage was obtained by degrading the pit membranes in control (Fig. 6C). Significant inhibition on fungal hyphae penetration was observed in the inner surface of the treated samples (Fig. 6D).

Discussion

Microbial genus *Bacillus* is producing several lipoproteins such as surfactin, iturin, and fengycin with potential antifungal activity (Ongena and Jacques, 2007; Romero *et al.*, 2007; Volpon *et al*., 2007). The production of surfactin was identified in *B. licheniformis* and iturin was observed in *B. subtilis* (Jacques *et al.*, 1999; Peypoux *et al*., 1999).

B. licheniformis producing secondary metabolite surfactin was also used as antitumoral, antiviral, and anti-mycoplasma agent (Peypoux *et al*., 1999). Biosynthesis of lipopeptides was catalyzed by non-ribosomal enzymes consisting of more than three subunits (Peypoux *et al*., 1999; Ongena and Jacques, 2007). Some researchers were already reported the antagonistic activity of some *Bacillus* strains against wood contaminating fungi *Aspergillus awamori*, *A. glaucus*, *A. fumigatus*, *A. pullulans*, *Choetomium* sp., *Penicillium variotii*, *P. variabile*, *P. expansum*, *Trichoderma* sp., and *T. harzianum* (Bernal *et al.*, 2002; Feio *et al.*, 2004). Our current research work is a search to identify the antifungal lipopeptides from *B. subtilis* and *B. licheniformis* with high potential activity against wood staining fungi *O. polonicum*, *O. flexuosum*, *O. tetropii*, and *O. ips*. The antagonistic activity of present study strains *B. licheniformis* and *B. subtilis*, were confirmed in 2% MEA agar plates against wood staining fungi. The

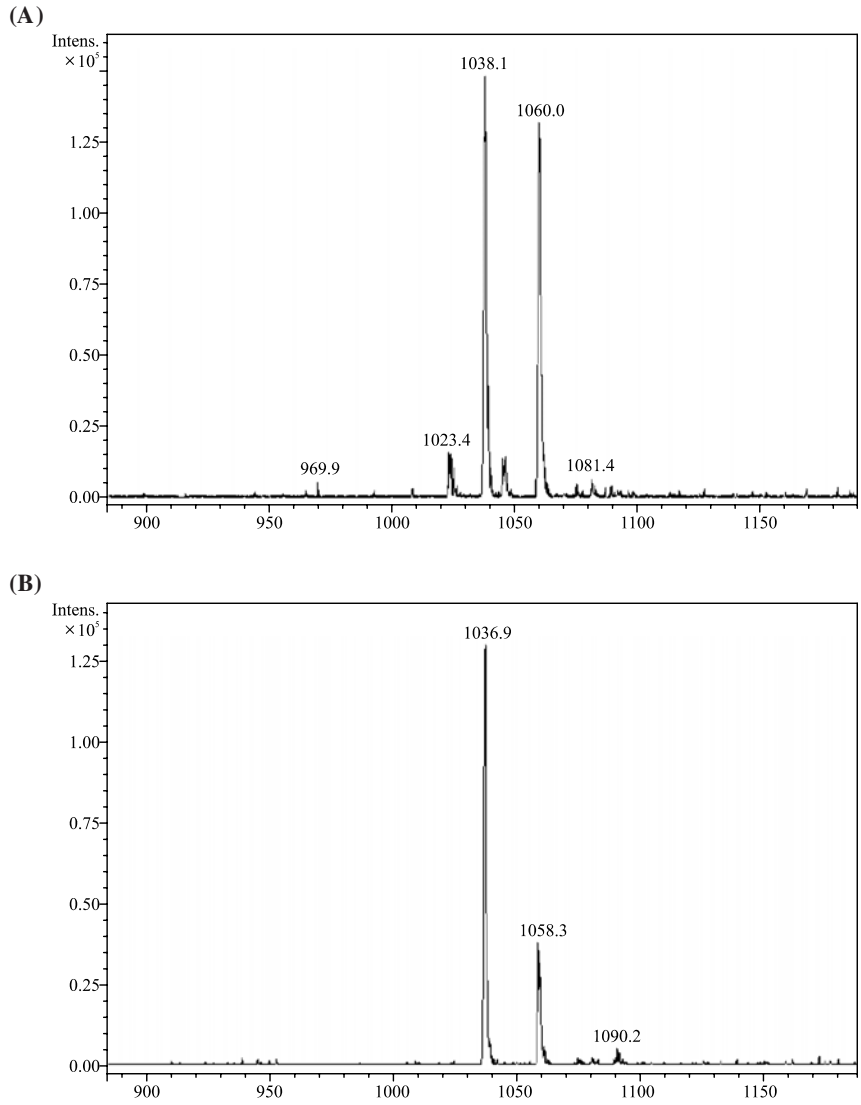

Fig. 5. MS spectrum of partially purified compounds of *B. licheniformis* EF 617325 (A) and *B. subtilis* EF 617317 (B).

lipoproteins were generally synthesized during growth phase in both wild and mutant strains of Bacilli (Haavik and Thomassen, 1973; Barr, 1975; Bernal *et al.*, 2002), normally the secondary metabolites were synthesized during 3rd to 5th day of incubation, but the maximum concentration of synthesized lipoproteins were obtained in exponential growth phase of *Bacillus* (Bernal *et al.*, 2002), i.e., during 7th to 10th day of incubation. Our experimental results about the duration for the complete production of lipopeptides also reflected the same results as published earlier (Haavik and Thomassen, 1973; Barr, 1975; Bernal *et al.*, 2002). The secondary metabolites produced in Luria-Bertani (LB) medium were synthesized without any additional carbon sources, such as mannitol, fructose, and sucrose. The cell free

Table 3. Fungal staining severity on control and treated wood blocks of *P. radiata* and *P. densiflora*

Fungal samples	Number of replicates	*P. radiata* Control		*P. radiata*				*P. densiflora* Control		*P. densiflora*			
				B. subtilis		*B. licheniformis*				*B. subtilis*		*B. licheniformis*	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
O. polonicum	8	3.1250	0.3536	2.2500	0.4629	2.1250	0.3536	3.8750	0.3536	1.3750	0.5175	1.2500	0.4629
O. flexuosum	8	4.000	0	3.1250	0.3536	3.7500	0.4629	4.000	0	3.000	0	3.000	0
O. tetropii	8	3.2500	0.4629	2.500	0.5345	3.5000	0.7559	3.6250	0.5175	2.2500	0.7071	2.5000	0.5345
O. ips	8	4.000	0	3.3750	0.5175	1.7500	0.4629	3.7500	0.4629	1.1250	0.3536	1.3750	0.5175

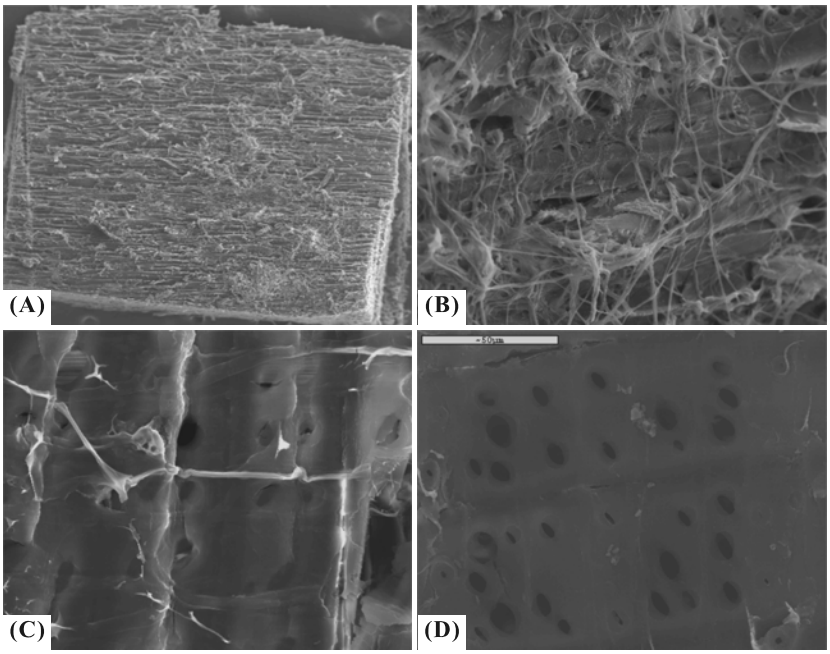

Fig. 6. Scanning Electron Microscopy photographs of inside of the longitudinal sections of wood blocks inoculated with *O. flexuosum*: Inside longitudinal section of wood block (control block [bar in scale~1,000 μm]) (A), Fungal hypha growth on wood block surface (control block [bar in scale~1,000 μm]) (B), Fungal hypha penetration in pit membranes (control blocks [bar in scale~50 μm]) (C), Uncontaminated pit membranes (treated blocks [bar in scale~50 μm]) (D).

fractions of active supernatants from *B. licheniformis* and *B. subtilis* strongly inhibited mycelial growth of the sapstaining fungal at the laboratory level. Thermostability and pH stability of the active supernatant fractions were stable up to boiling point 100°C and compounds having stable antagonistic activity in both acidic and alkaline conditions. These results also reflected the results which have already been reported about the thermostability and pH stability of lipopeptides synthesized by *Bacillus* sp. The structural surface group of active supernatant fraction of both *B. subtilis* and *B. licheniformis* were investigated by FT-IR. Strong characteristics bands were appeared from 1655 to 1635 cm^{-1} in both bacterial fractions. The strong peaks observed at 1655 cm^{-1} in *B. subtilis* (Fig. 2B) and 1659 cm^{-1} in *B. licheniformis* were assigned for amide I band (C-O-N bond) (Fig. 2A). 1535 cm^{-1} peak of *B. subtilis* (Fig. 2B) and 1540 cm^{-1} peak of *B. licheniformis* (Fig. 2A) were attributed for the C-N stretching mode (amide II band) combined with the deformation mode of the N-H bond (Cho *et al*., 2003; Romero *et al*., 2007). The strong bands were observed at the range from 2856 to 2959 cm^{-1} in *B. subtilis* (Fig. 2B) and 2929 to 2962 cm^{-1} in *B. licheniformis* (Fig. 2A), indicating the typical CH stretching vibration in the alkyl chain (Romero *et al*., 2007). The peak was observed at 1725 in *B. licheniformis* (Fig. 2A) due to the lactone carbonyl absorption typical for surfactin family of lipopeptides (Romero *et al*., 2007). The broad peaks observed around 3300 cm^{-1} indicated the presence of N-H groups in the molecules (Cho *et al*., 2003; Romero *et al*., 2007). The active metabolites of *B. licheniformis* and *B. subtilis* were partially purified using anion exchange chromatography followed by Sephadex gel filtration chromatography. Partially purified compounds significantly inhibited the sapstaining fungal growth. The partially purified compounds of both *B. subtilis* and *B. licheniformis* were identified by mass spectrometry and these peaks were belongs to surfactin and iturin groups. The average molecular weight of lipopeptides segregated by *Bacillus* sp., were less than 2000 Da. Four major ion peaks were detected between *m*/*z* 1023 to 1081 in *B. licheniformis* (Fig. 5A), and three major ion peaks were detected at between *m*/*z* 1036 to 1090 in *B. subtilis* (Fig. 5B), these peaks belongs to surfactin and iturin groups, respectively. Some researchers have already proved the antifungal activity of surfactin and iturin groups produced by *Bacillus* sp., and those molecular mass numbers were reported as between *m*/*z* 1008 to 1098 (Romero *et al*., 2007). The present result also suggested the surfactin and iturin molecular mass as between *m*/*z* 1023 to 1090. All partially purified cyclic lipopeptides are sharing the general amino acid sequences, composed of seven ά-amino acids (mainly Pro, Asn, Ser, Tyr, and Gln) and one β-amino fatty acid (Cho *et al*., 2003). This cyclic lipopeptides consisting of β-hydroxy fatty acid is incorporated into a peptide moiety and has a specific activity against filamentous fungi, in addition, the antifungal activity also rising by phospholipids, sterols, oleic acids and unsaturated fatty acids (Vanittanakom *et al*., 1989). Moyne *et al*. (2001) also suggested that antagonistic activity of lipopeptides may rise by the length of the β-amino acids. Another cyclic lipopeptide belongs to the family of surfactin, consisting of heptapeptides containing a β-hydroxy fatty acid and this group showed a strong antago-

nistic activity against fungi when combined with iturin A (Romero *et al*., 2007). The antifungal activity of lipopeptides depends on the following reasons: sterol composition of fungal plasma membrane, formation of lipid and peptide complex, membrane permeability and possibilities of membrane pore size increased by lipopeptide. These reasons suggested that the antagonistic activity of lipopeptides not only due to its chemical composition. As well the sterol concentration of fungal plasma membrane is higher than that of yeasts, thus the lipopeptides are highly active against fungi than yeasts.

Many researchers reported the field trials of biocontrol of wood decay and wood staining fungi using bacteria and fungi (Barr, 1975; Bernal *et al.*, 2002; Feio *et al.*, 2004). Even though, in most of the reports the living microorganisms only tested under *in vivo* condition, there were no strong previous reports about the inhibition potential of purified or partially purified form of biological antagonistic compounds in *in vivo* condition. The living microorganisms can easily contaminate the surface of wood samples. In this paper, we used the non-cellular part (active supernatants) for *in vivo* experiments. Based on our results, the penetration of active compounds (lipopeptides) into wood elements was significant and these can suppress the internal fungal decay in the centre region of wood samples. This paper provided the experimental evidence concerning the antifungal ability of partially purified compounds of *Bacillus* sp. against wood staining fungi and the responsible compounds were identified as surfactin and iturin. Further investigations are currently conducted to find out the efficacy of bio-compounds as an antifungal agent on wood in the field. In conclusion, this report enhanced the possibilities of biological compounds to be used as bio-protective agents in wood against sapstaining fungi. However, further studies will be necessary to improve the antifungal potential of bio-compounds on wood in the field. Modification and mutations in surfactin and iturin could improve its antifungal potential in the field. These compounds could be a very good substitution for chemicals which are currently used to control sapstaining fungi in the wood industries.

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