

ABILITY OF SELECTED BACTERIAL AND FUNGAL BIOPROTECTANTS TO LIMIT FUNGAL STAIN IN PONDEROSA PINE SAPWOOD¹

Bernhard Kreber

Graduate Research Assistant

and

J. J. Morrell

Assistant Professor

Department of Forest Products

Oregon State University

Corvallis, OR 97331

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ABSTRACT

The ability of 15 bacterial and fungal isolates to inhibit fungal stain of ponderosa pine sapwood was studied on small wood samples exposed in a moist environment. Several isolates including *Bacillus subtilis* were capable of inhibiting fungal stain although the protective effect was lost upon prolonged exposure. More detailed evaluations of two *B. subtilis* isolates revealed that performance was closely tied to the media on which the organisms were grown prior to wood application, with the optimal growth media appearing also to be associated with the highest degree of stain protection. Further evaluation of *B. subtilis* 733 A for evidence of production of water-soluble or volatile antibiotics suggested that antibiotics were not present on the wood, although further studies on bioprotectant mechanisms are suggested.

Keywords: Bioprotection, *Bacillus subtilis*, fungal stain, antibiotics.

INTRODUCTION

Traditionally, chemical treatments have been used to protect unseasoned lumber from biological discoloration during storage and transportation. However, potentially hazardous health and environmental effects have brought the use of some antistain chemicals into question and indicate a need for new approaches to biological stain prevention.

Biological protection or *bioprotection* is one alternative to chemical treatments. Bioprotection uses one organism to inhibit the growth or damaging effects of another. To date, bioprotection has been studied primarily for its

ability to prevent fungal degradation of wood products (Ricard 1966; Ricard and Bollen 1968; Bruce and King 1983, 1986a, b; Bruce et al. 1990, 1991; Bruce and Highley 1991) or to eliminate incipient decay (Ricard et al. 1969; Morris and Dickinson 1981; Highley 1989); and has received much less attention as a protectant against wood discoloration. However, several studies on the use of bacteria and fungi (Benko 1986, 1988; Seifert et al. 1988) and other antimicrobial agents produced in a bioprotectant culture fluid (Benko 1989; Croan and Highley 1991a, b; Highley et al. 1991) to protect unseasoned lumber from biological discoloration have recently been published.

In this study, 15 bacterial and fungal isolates were screened for their ability to prevent microbial discoloration on unseasoned ponderosa pine (*Pinus ponderosa* Laws.) sapwood. In

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addition, one of these microorganisms, *B. subtilis*, was further examined for the effects of media composition on its bioprotective capacities, its antibiotic production, and its effect on spore germination.

MATERIALS AND METHODS

Initial screening trials

The bioprotectants evaluated in the initial screening trials are listed in Table 1, and the mold and stain fungi used for testing (selected from the Forest Research Laboratory culture collection, Oregon State University, Corvallis) are recorded in Table 2. The bioprotectant cultures were maintained on agar slant tubes with the appropriate growth media (Table 1), and the mold and stain fungi were maintained on malt agar (MA) (1.5% malt extract, 1% agar) plates. All cultures were stored in a cold room (0 C) until needed. Chemicals used for media composition were purchased from Difco Laboratories (Detroit, MI) or Sigma Chemical Company (St. Louis, MO).

Preparation of bioprotectant suspensions.—Bacterial bioprotectant suspensions were prepared by transferring the bacterial inocula into 250-ml Erlenmeyer flasks containing 50 ml sterile (121 C) liquid growth media of the appropriate type (Table 1) and incubating the flask contents at room temperature (23 C) on a rotary shaker (manufactured by Lab Line Instr. Inc., Melrose Park, IL) at 80 RPM for approximately 65 hours. The resulting cultures were aseptically harvested by draining the flask contents into 400-ml aluminum trays and adding 200 ml sterile (121 C, 45 min) distilled water to produce 250-ml suspensions. To prepare the fungal bioprotectant suspensions, the test fungi were incubated at 27 C on MA plates (or, for *S. cerevisiae*, at room temperature on malt extract in a flask) until abundant sporulation developed. Each plate was then flooded with 4 ml distilled water, and the spores and hyphal fragments that rose to the water's surface were harvested and placed in 400-ml aluminum trays, and enough distilled water was added to each tray to produce 250-ml suspensions.

Preparation of fungal stain suspensions.—

The mold and stain fungi listed in Table 2 were added to 250-ml Erlenmeyer flasks containing 50 ml sterile (121 C, 20 min) 1% malt extract media and incubated at room temperature (23 C) on a rotary shaker at 80 RPM until an abundant dark pigmented mycelial biomass developed. The contents of each flask were then vacuum filtered (1 bar) through Whatman #4 filter paper with a Buchner funnel, and the collected spores and hyphal fragments were rinsed with distilled water off the filter paper and into a single 500 ml beaker. The combined biomass in the beaker was then blended for about 10 seconds, and enough distilled water was added to the beaker to obtain 1 liter of suspension. This suspension was then drained into a squeeze bottle and kept at room temperature until needed. Additional fungal biomass, prepared as described above, was added to the fungal stain suspension at approximately 2-week intervals to maintain fungal viability.

Bioprotectant testing.—For each bioprotectant tested, 20 steam sterilized (100 C, 15 min) ponderosa pine specimens (0.5 × 1.0 × 3.0 cm) were placed in a 400-ml aluminum container, covered with weighted wire to keep them submerged during treatment, and flooded for about 8 minutes with 250 ml of the bioprotectant suspension. While all replicates were prepared at the same time, inoculum density was not quantified. An additional set of ponderosa pine specimens, used as the control, was flooded with 250 ml distilled water for the same time period. The containers were then drained and the specimens were removed, allowed to surface dry for approximately 2–3 minutes, and sprayed on both wide faces to the point of runoff with the prepared fungal stain suspension. The specimens were then incubated employing a method described by Seifert et al. (1988). For each bioprotectant, five replicate tests were conducted. For each test, four wood specimens treated with both bioprotectant and stain fungi, and one control specimen treated with stain fungi only, were placed on glass rods in a sterile glass petri dish containing three filter paper discs, and 5 ml distilled water was

TABLE 1. *Bioprotectants evaluated on ponderosa pine.*

Species	Strain number	Source	Growth medium
Bacteria			
<i>Bacillus subtilis</i> Cohn	733 A	Forintek Canada Corp., Ottawa, Canada	Nutrient broth (NB) (0.3%)
<i>Bacillus subtilis</i> Cohn	Quantum 4000 HB	J. Loper, USDA Agric. Research Service (ARS), Corvallis, OR	NB
<i>Pseudomonas cepacia</i> Burkholder		T. Highley, Forest Products Laboratory, Madison, WI	Kings B Media (KB) (2% proteose peptone, 1% glycerol, 0.15% potassium phosphate monobasic, 0.15% magnesium sulfate)
<i>Pseudomonas fluorescens</i> Migula	Pf-5 (#3832)	J. Loper, ARS, Corvallis, OR	KB
<i>Pseudomonas putida</i> Migula	A-12	J. Loper, ARS, Corvallis, OR	KB
<i>Pseudomonas putida</i> Migula	N-1R	J. Loper, ARS, Corvallis, OR	KB
<i>Streptomyces tendae</i>	695A	Forintek, Ottawa, Canada	Potato-Dextrose-Broth (PDB)
<i>Streptomyces</i> sp.	C 248	Forintek, Ottawa, Canada	PDB
<i>Streptomyces</i> sp.	C 254	Forintek, Ottawa, Canada	PDB
Fungi			
<i>Gliocladium aurifilum</i>	675 A	Forintek, Ottawa, Canada	Malt agar (MA) (1.5% malt extract, 1% agar)
<i>Gliocladium virens</i> J. H. Miller, J. E. Giddens, & A. A. Foster	H 3	J. Loper, ARS, Corvallis, OR	MA
<i>Penicillium thomii</i> Maire	655 A	Forintek, Ottawa, Canada	MA
<i>Saccharomyces cerevisiae</i> Meyen ex Hansen	591 A	Forintek, Ottawa, Canada	MA
<i>Trichoderma harzianum</i> Rifai	25	Forest Research Lab (FRL), Oregon State University, Corvallis	MA
<i>Trichoderma harzianum</i> Rifai	142	FRL, Oregon State University, Corvallis	MA

added. For additional controls, specimens treated with stain fungi or bioprotectant only were placed in separate petri dishes.

The dishes were coded independently to minimize evaluation bias and were then incubated in the dark at 27 C for 4 weeks; deionized water was added as necessary to maintain a moist environment. The incubated wood

samples were examined weekly and, after 4 weeks, were evaluated with methods described in the next section.

Evaluation of bioprotectants.—At the end of the 4-week incubation period, average degree of surface stain (ADS) was determined, and percentage of fungal stain inhibition (FSI) was calculated for each of the bioprotectants tested.

TABLE 2. Mold and stain fungi used to evaluate bioprotectant capabilities on ponderosa pine sapwood.

Species	Strain number
<i>Alternaria alternata</i> (FR:FR) Keissl.	ED 113
<i>Aspergillus niger</i> van Tieghem ^a	BOB
<i>Aureobasidium pullulans</i> [de Bary] G. Arnaud ^a	Zm 3
<i>Bispora betulina</i> (Corda) S. J. Hughes	1
<i>Ceratocystis pilifera</i> (Fries) C. Moreau ^b	55 A
<i>Cladosporium elatum</i> (C. Harz) Nannf.	563-I-1
<i>Hormoconis resiniae</i> (Lindau) Arx & G. A. De Vries	P 1600
<i>Penicillium frequentans</i> Westling ^a	43
<i>Phialophora fastigata</i> (Lagerberg & Melin) Conant	14
<i>Rhinocladia atrovirens</i> Nannf. in Melin & Nannf.	68
<i>Ulocladium chartarum</i> (G. Preuss) E. Simon	100

^a Not included in suspension prepared for antibiotic production test.

^b Not included in fungal stain suspension used for initial screening trials.

The following modified grading system proposed by Benko (1988) was used to visually quantify ADS:

- 0 = unstained wood
(no visible signs of stain)
- 1 = incipient establishment of microbial growth
(evidence of microbial growth on surface)
- 2 = moderate stain
(at least 25% of the wood surface stained)

- 3 = heavily stained
(more than 50% of the wood surface stained)

Percentage of FSI was calculated with the following equation:

$$[1 - (\text{ADS}/\text{ADS}_{\text{control}})] \times 100 \quad (1)$$

ADS and ADS_{control} are the average degree of stain on treated and control specimens, respectively.

Effects of growth media on bioprotectant performance

Bacillus subtilis #733 A and *B. subtilis* ATCC 6633 were selected to quantify the effects of different growth media on bioprotectant performance against stain fungi. Most of these growth media had been used in prior studies, for example, for antibiotic production (Kugler et al. 1990). The two bioprotectants were cultured in 1.5 liters of each test medium (Table 3) except for 0.3% nutrient broth; for this medium, the bioprotectants were cultured in six 250-ml Erlenmeyer flasks containing 50 ml sterile nutrient broth, and the resulting 300 ml of culture were added to 1.2 liters distilled water to produce 1.5 liters of fluid.

Treatment of wood with bioprotectant culture fluids and fungal stain suspension.—The same wood-treatment procedures were used for this experiment as for the initial screening trials, except that wood specimens for this test measured 0.5 × 1.5 × 3.0 cm, an 8-week incubation period was used, and one half of the treated specimens were resprayed with the fun-

TABLE 3. Culture conditions of growth media used to evaluate the effect of different media on bioprotectant performance against wood-staining fungi.

Medium	Culture conditions			Reference
	Time (hr)	Temp (°C)	RPM	
Nutrient broth (NB) (0.3 or 1.5%)	60	25	80	Claus and Fritz 1989
PA media	18	37	225	Ozcengiz et al. 1990
Penassay broth (PB)	48	30	250	Bernier et al. 1986
PL media	48	25	120	Kugler et al. 1990
Tryptic soy-broth (TSB)	72	37	250	Anker et al. 1948
Yeast-dextrose-carbonate (YDC)	72	25	80	Wilson et al. 1967
Yeast-malt-dextrose broth (YMBD)	25	25	80	Seifert et al. 1987

gal stain suspension at 2-week intervals to simulate repeated influx of staining organisms.

After 8 weeks, the effect of different media on the two bioprotectants' performance was assessed. Average degree of surface stain (ADS) and percentage of fungal stain inhibition (FSI) were determined as for the initial screening trials. Wood specimens were then split longitudinally, and the average degree of internal stain (ADIS) was determined based on the following grading system:

- 0 = unstained wood (no visible signs of internal stain)
- 1.0 = small (1- or 2-mm diameter) stained spots visible near the end grain
- 1.5 = thin (0.5 mm) stained shell around no more than 25% of the circumference
- 2.0 = stained shell (0.5 mm) around no more than 50% of the circumference
- 2.5 = stained shell (0.5 mm) around more than 50% of the circumference
- 3.0 = stained shell (0.5 mm) around the entire circumference
- 4.0 = more than 50% of the internal wood surface area stained

Percentage of internal fungal stain inhibition (IFSI) was calculated as for FSI [Eq. (1)] but using the ADIS values.

Antibiotic production

Antibiotic production represents one potential bioprotection mechanism and was investigated for *B. subtilis* #733 A with the following procedure. Ponderosa pine specimens treated for 8 minutes with the 0.3 NB *B. subtilis* #733 A suspension and sprayed with the fungal stain suspension, and control specimens treated only with the bioprotectant, fungal stain suspension, or 300 ml distilled water, were incubated at 27 C as for the initial screening trials.

After 1, 2, 3, or 4 weeks of incubation, a series of concentrated extracts was prepared from the treated and control specimens as follows. Ten specimens were removed from the incubator, placed in two 250-ml Erlenmeyer flasks, each containing 50 ml 0.001% Tween 80 (Manooleate), and incubated on a rotary

shaker at 120 RPM for 10 hours at room temperature. The resulting extracts in the two flasks were combined and filtered through a sequence of 0.8-, 0.45-, and 0.22- μ membranes. The filtrate was then concentrated in an Amicon Stirred Ultra-Filtration Cell with Amicon Diaflo YM 3 and YM 10 membranes (all manufactured by Amicon Inc., Danvers, MA), to produce concentrated extracts with molecular weight cutoffs of <3,000, 3,000–10,000, and >10,000. The concentrated extracts were then stored at 4 C until needed.

Antibiotic production in each extract was tested by pipetting a small amount (0.05 ml) of the extract into a well (0.4 cm in diameter, 0.3 cm deep) cut into the center of an MA petri dish. The petri dishes were then abundantly seeded with a fungal stain suspension prepared as for the initial screening trials but containing a slightly different combination of fungi (see Table 2). The seeded MA dishes were incubated for 7 days in the dark at 26 C and then were examined for evidence of zones of inhibition around the well. All tests were performed in two replicates. In addition, as controls for each test, one MA dish received only the 0.05 ml of concentrated extract and another was seeded with the fungal stain suspension only.

Bioprotectant effect on spore germination

Inhibition of spore germination is critical for successful protection against wood-staining fungi. This test compared fungal spore germination in the presence and absence of *Bacillus subtilis* #733 A. Steam sterilized ponderosa pine specimens (0.45 \times 1.4 \times 1.5 cm) were dipped into 250-ml *Bacillus subtilis* #733 A suspension (prepared as described for the initial screening trials) or, for the controls, into 250 ml sterile distilled water, for approximately 8 minutes. Samples were then allowed to surface dry for 2–3 minutes prior to being sprayed to runoff with *A. niger* spores that had been cultured on PDA and flooded from the mycelial mat with 0.001% Tween 80. The treated wood specimens were then placed in an 8.2- \times 3.0- \times 1.0-cm aluminum incubation

chamber with a 6.6- × 1.4- × 0.5-cm molded depression and covered with a glass slide. The incubation environment was sealed with mineral oil, which allowed oxygen to diffuse into the chamber but prevented moisture loss from the chamber and restricted entry of competing microbes.

The specimens were incubated at room temperature for 6, 8, 10, 12, 24, or 36 hours. Then, a 20- μ -thick section was cut from each specimen with a Spencer microtome (manufactured by Spencer Lens Co., Buffalo, NY), stained with 1% safranin O followed by steaming picocyanine blue according to the procedure described by Wilcox (1964), and mounted in water. A Leitz microscope at 10 \times magnification was then used to examine each section for spore germination as evidenced by production of a 2- to 3- μ -long germ tube. Percentage of spore germination per square centimeter of wood surface area was calculated with the following equation:

$$[(A/B)/C] \times 100 \quad (2)$$

where:

- A = mean of three replicate conidia germination counts at a selected time
- B = potential germination based on mean number of colony-forming units produced on PDA
- C = surface area of ponderosa pine specimens

RESULTS AND DISCUSSION

Initial screening trials

The most effective bioprotectant was *Bacillus subtilis* isolate 733 A, which completely prevented fungal discoloration on ponderosa pine sapwood over a 4-week period (Fig. 1a), producing 89% fungal stain inhibition in comparison with the control (Table 4). However, after an additional 4 weeks, *B. subtilis* #733 A showed a gradual decrease in antagonistic activity against mold and stain fungi (Fig. 1b). This result suggests that *B. subtilis* #733 A delayed but could not completely prevent fungal invasion of unseasoned ponderosa pine sap-

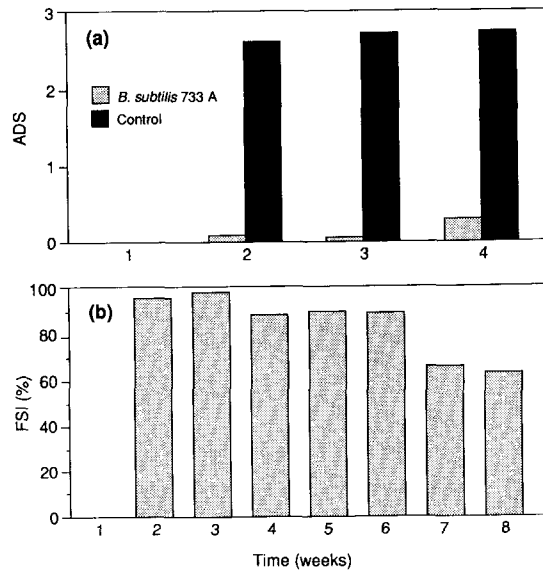


FIG. 1. Bioprotection associated with application of *B. subtilis* #733 A as (a) average degree of surface stain (ADS) and (b) percentage of fungal stain inhibition (FSI). ADS for sprayed specimens at 32 C was 0.

wood, possibly because of incomplete substrate colonization (Seifert et al. 1987).

Competition for nutrients and space, and/or antifungal compounds produced in *B. subtilis* culture fluids, have been suggested as probable modes of action of *B. subtilis* isolates against wood-staining microorganisms (Bernier et al. 1986; Florence and Sharma 1990). However, these hypotheses were drawn either from culture analysis or from short-term (2-week) stain tests on rubberwood, and so their relevance to longer protective periods is questionable. To accurately assess the microbial activity of bioprotectants in regard to wood-staining microorganisms, the activity should be evaluated exclusively on wood substrates and for longer time periods.

Although *Gliocladium virens* #H-3 reduced fungal stain by 62.96% in comparison with the control, it also produced an extensive greenish aerial mycelium and spores on the wood surfaces that were detrimental aesthetically. Seifert et al. (1988) reported similar effects with *G. roseum* when evaluating it as a potential bioprotectant. Unless *G. virens* is found to pro-

TABLE 4. Average degree of surface stain (ADS) and percentage of fungal stain inhibition (FSI) for bioprotectants 4 weeks after wood treatment.

Species	ADS of treated specimens	ADS of control specimens	FSI ^a (%)
<i>B. subtilis</i> 733 A	0.30 ^b (0.46) ^c	2.70 ^d (0.69)	88.88
<i>B. subtilis</i> Q 4000 HB	2.60 (0.74)	2.60 (0.80)	-1.92 ^e
<i>P. cepacia</i>	2.70 (0.64)	2.80 (0.60)	-3.57
<i>P. fluorescens</i> Pf-5	3.00 (0.00)	2.40 (0.92)	-25.00
<i>P. putida</i> A-12	1.70 (0.84)	2.60 (0.80)	34.62
<i>P. putida</i> N-1R	2.70 (0.64)	2.80 (0.60)	-3.57
<i>Strept. tendae</i> 695 A	2.30 (0.95)	2.80 (0.60)	17.82
<i>Strept. sp.</i> C 248	3.00 (0.00)	3.00 (0.00)	0.00
<i>Strept. sp.</i> C 254	2.75 (0.62)	3.00 (0.00)	8.33
<i>G. aurifilium</i> 675 A	2.75 (0.62)	2.90 (0.30)	5.17
<i>G. virens</i> H 3	1.00 (0.77)	2.77 (0.64)	62.96
<i>P. thomii</i> 655 A	1.80 (0.92)	1.40 (0.80)	-28.57
<i>S. cerevisiae</i> 591 A	2.15 (0.85)	2.30 (0.64)	6.52
<i>T. harzianum</i> 25	2.45 (0.86)	2.80 (0.60)	12.50
<i>T. harzianum</i> 142	2.70 (0.64)	2.40 (0.91)	-12.50

^a % FSI = $[1 - (ADS/ADS_{control})] \times 100$.

^b ADS values represent mean of 20 replicates.

^c Values in parentheses represent standard deviations.

^d ADS_{control} values represent mean of 10 replicates.

^e Negative FSI values denote increased staining compared to controls.

duce antifungal compounds that are particularly effective against the mold and stain fungi prevalent on unseasoned sapwood, this species is probably not a viable bioprotectant against fungal stain.

With a 34.62% reduction in fungal stain compared to the control, *Pseudomonas putida* #A-12 also exhibited some potential for bioprotection of unseasoned lumber, probably by competing with stain fungi for nutrients and space or by antibiosis. Fluorescent *Pseudomonas* spp. are able to control pythopathogens through both antibiosis (Thomashow et al. 1990) and iron competition (Loper 1988), and, with the latter mechanism, to reduce chlamyospore germination in the rhizosphere of plants (Elad and Baker 1985).

Ponderosa pine specimens treated with *Trichoderma harzianum* #25 contained a yellow stain and conspicuous green spores starting at 2 weeks after treatment, a point at which most control specimens had already exhibited progressive stain patterns. Although the yellow stain was aesthetically detrimental, mold and stain fungi growth was not observed on the wood, suggesting the presence of antimicrobial secondary metabolites in or associated with

the yellow stain. Previous studies have reported *Trichoderma* spp. production of volatile and nonvolatile antibiotics that inhibit selected decay fungi (Dennis and Webster 1971a, b; Bruce et al. 1984).

The failure of the remaining tested bioprotectants to prevent microbial stain on ponderosa pine specimens may reflect inappropriate growth media, adverse environmental conditions, and/or the inability of these species to compete with microbial competitors once they became established on the wood substrate. Klingström and Johanssen (1973) noted that different microbial strains within the same species can produce markedly different results. This observation was borne out in our study by the different results for *B. subtilis* isolates 733 A and Q 4000 HB.

Effect of growth media on bioprotectant performance

Incubation of the two *B. subtilis* isolates (733 A and ATCC 6633) on eight types of media prior to wood application resulted in dramatically different degrees of stain prevention. Generally, the best results were obtained when the isolates were incubated on their preferred

TABLE 5. Percentages of surface and internal fungal stain inhibition (FSI and IFSI)^a achieved with *B. subtilis* #733 A after 8 weeks of incubation on various media.

Medium	Temperature (°C)					
	32		26		20	
	Resprayed ^b	Not resprayed ^c	Resprayed	Not resprayed	Resprayed	Not resprayed
1.5 NB	-5.50 (-87.69)	-31.81 (-22.58)	13.33 (11.42)	11.66 (68.18)	5.0 (47.54)	16.66 (63.63)
0.3 NB	0.00 (5.60)	27.58 (54.74)	38.88 (43.0)	55.76 (86.84)	-14.58 (28.00)	14.00 (96.66)
PB	3.66 (-83.07)	4.16 (-150.0)	16.66 (N.D.) ^d	1.66 (N.D.)	8.33 (96.06)	6.60 (N.D.)
YMDB	-9.25 (-167.0)	-46.66 (-530.0)	0.00 (5.41)	0.00 (9.37)	1.66 (9.18)	1.66 (8.33)
YDC	0.00 (-203.0)	0.00 (-94.73)	0.00 (3.20)	0.00 (3.92)	0.00 (34.78)	0.00 (10.98)
TSB	0.00 (-940.0)	0.00 (-500.0)	0.00 (-9.77)	0.00 (-40.0)	0.00 (-21.00)	0.00 (-20.0)
PA	10.00 (40.00)	12.50 (44.61)	0.00 (17.31)	0.00 (20.60)	0.00 (9.23)	0.00 (9.70)
PL	-22.91 (-71.42)	-11.11 (-460.0)	1.66 (13.46)	0.00 (-11.11)	0.00 (32.00)	0.00 (32.00)

^a FSI and IFSI values calculated as $[1 - (\text{ADS}/\text{ADS}_{\text{control}})] \times 100$. IFSI values are in parentheses. Negative values denote increased staining compared to controls.

^b After initial treatment, specimens were resprayed at 2, 4, and 6 weeks with fungal stain suspension.

^c Specimens were sprayed with fungal stain suspension during initial treatment only.

^d Not determined because treated and control specimens exhibited no signs of internal stain.

medium (0.3 NB for *B. subtilis* #733 A, and Penassay broth for *B. subtilis* ATCC 6633) (Tables 5 and 6). Between the two isolates grown on their preferred media, the combined FSI and IFSI data showed *B. subtilis* #733 A to be the superior bioprotectant of unseasoned ponderosa pine sapwood.

As well as growth media, the incubation temperature of the treated wood specimens and the number of times the specimens were sprayed with fungal stain suspension also affected the performance of *B. subtilis* #733 A (Fig. 2). Favorable incubation temperatures (26 and 32 C) may have enabled the bioprotectant rapidly to exploit readily available nutrients, thereby excluding microbial competitors. Another possibility is that any temperature-sensitive antifungal compounds produced in the culture broth of the biological agent may have remained active for a longer time period at these temperatures. The consistent decrease in protection with respraying may be due to a

gradual loss of antibiotic from the wood surface, which enabled the resprayed spores to germinate more easily.

Most of the media evaluated had no significant effect on bioprotectant results, perhaps due to inappropriate culture conditions. Benko and Highley (1990a, b) reported that media composition significantly affects the activity of fungitoxic bacterial compounds used for wood treatment. However, their studies tested these compounds against only two wood-staining fungi, *Ceratocystis coerulea* (Münch) Bakshi and *Trichoderma harzianum* Rifai. These two fungi might behave differently in competition with other sapwood-staining fungi such as those used in the current study.

Antibiotic production

None of the extracts prepared from the treated wood specimens inhibited growth of mold and stain fungi in comparison to the controls. Either antibiotics were not produced, an in-

TABLE 6. Percentages of surface and internal fungal stain inhibition (FSI and IFSI)^a achieved with *B. subtilis* ATCC 6633 after 8 weeks of incubation on various media.

Medium	Temperature (°C)					
	32		26		20	
	Resprayed ^b	Not resprayed ^c	Resprayed	Not resprayed	Resprayed	Not resprayed
1.5 NB	5.00 (70.73)	0.00 (20.00)	6.66 (16.84)	0.00 (58.06)	5.00 (59.58)	0.00 (14.76)
0.3 NB	15.00 (90.00)	11.53 (33.33)	3.33 (48.42)	0.00 (21.25)	0.00 (17.14)	0.00 (36.11)
PB	5.00 (75.00)	27.00 (90.00)	16.66 (73.58)	13.30 (97.61)	18.51 (92.41)	1.66 (16.82)
YMDB	-13.88 (N.D.) ^d	30.00 (0.00)	3.33 (24.19)	11.66 (53.48)	0.00 (14.75)	0.00 (25.00)
YDC	-20.00 (-84.21)	-7.14 (-414.0)	0.00 (-27.55)	0.00 (-25.00)	0.00 (-12.54)	0.00 (-13.72)
TSB	-11.11 (-275.0)	-87.50 (-205.0)	0.00 (-15.00)	0.00 (-1.88)	-3.44 (4.72)	0.00 (-4.61)
PA	11.11 (53.63)	10.71 (12.72)	0.00 (6.45)	0.00 (14.72)	0.00 (8.82)	0.00 (4.12)
PL	13.33 (-103.0)	1.66 (-13.33)	0.00 (-8.51)	0.00 (14.09)	0.00 (21.42)	0.00 (23.80)

^a FSI and IFSI values calculated as $[1 - (ADS/ADS_{control})] \times 100$. IFSI are in parentheses. Negative values denote increased staining compared to controls.

^b After initial treatment, specimens were resprayed at 2, 4, and 6 weeks with fungal stain suspension.

^c Specimens were sprayed with fungal stain suspension during initial treatment only.

^d Not determined because treated and control specimens exhibited no signs of internal stain.

appropriate extraction procedure deactivated them (for example, through pH changes or presence of proteolytic enzymes), or the solvent used for the extraction was unable to release the antibiotics from the wood substrate (Dawson-Andoh and Morrell 1991).

Our results suggest that competition for nutrients and space are the dominant modes of action in this bioprotectant system although assaying for specific antifungal compounds known to be produced by particular isolates of *B. subtilis* (Kugler et al. 1990) or refining the extraction procedure may have produced more precise results.

Bioprotectant effect on spore germination

In our test conditions, *B. subtilis* #733 A did not inhibit *A. niger* spore germination (Table 7). This result is consistent with that of Seifert et al. (1987), who also found, as we did, that the bioprotectant apparently colonized the wood substrate in clusters. In some instances, however, close spatial relationships such as

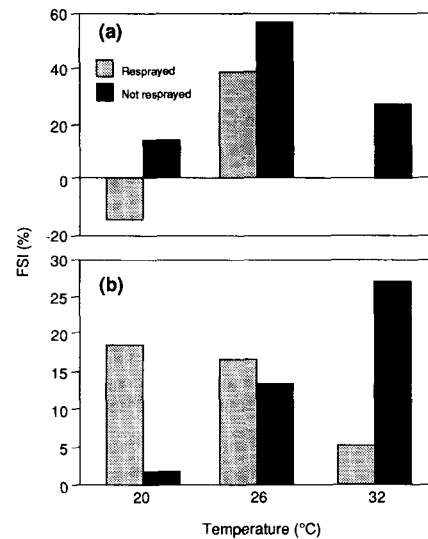


FIG. 2. Effects of optimal media on the ability of bioprotectants to protect ponderosa pine from microbial stain: (a) *B. subtilis* #733 A with 0.3 NB; (b) *B. subtilis* ATCC 6633 with PB.

TABLE 7. Effect of *B. subtilis* #733 A on germination of *A. niger* conidiospore germination on ponderosa pine sapwood at selected times.

Time ^b	Spore germination on wood specimens (%) ^a	
	Control	<i>B. subtilis</i> -treated
6	0.015	0.018
8	0.161	0.167
10	0.272	0.262
12	0.339	0.317

^a As a percent of *A. niger* spores initially applied.

^b Hours after spore application. After 24 hours of incubation spore generation was too dense to be quantified.

those described by Campbell (1989) between *B. subtilis* cells and swollen conidiospores were noted. In these cases, competition for nutrients may have been occurring, but the destructive sampling procedure we used prevented us from verifying this possibility.

Although *A. niger* may not be representative of all mold and stain fungi, its rapid growth rate is typical of these organisms and highlights the need for bioprotectants to be highly competitive for both nutrients and space. Modification of the wood substrate in favor of the bioprotectant, to promote uniform substrate colonization by the bioprotectant, may also be necessary (Dawson and Morrell 1990; Morrell 1990).

CONCLUSIONS

Of the bioprotectants we tested, *Bacillus subtilis* #733 A provided superior protection against wood-staining microorganisms on ponderosa pine sapwood, but only on optimized media at higher temperatures. Strategies such as nutritional selection (Benko and Highley 1990a, b) or modification of the wood substrate in favor of *B. subtilis* (Dawson-Andoh and Morrell 1990; Morrell 1990) may further improve the organism's bioprotective capabilities.

Although we found no evidence of antibiotic production with *B. subtilis*, it is possible that these compounds either were produced in very small quantities or were volatile, and thus were not detectable with our test methods.

The fact that *B. subtilis* #733 A did not inhibit spore germination suggests that this bio-

protectant is incapable of completely protecting ponderosa pine sapwood. However, spatial relationships between biological agents and other more important wood staining fungi need more detailed investigation over longer time periods than were used in this study.

Our results indicate that bioprotection against mold and stain fungi is a viable prospect. However, considerable basic research in areas such as optimizing growth of potential bioprotectants and determining the effect of environmental factors (temperature, wood moisture content) on bioprotection still needs to be done. Furthermore, a better understanding of microbial interactions on wood surfaces is essential before large-scale use of bioprotection is attempted.

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