AN ABSTRACT OF THE THESIS OF

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Abstract approved: _____
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Biological stain (blue stain) reduces wood value and prevents its use in many structural applications. Increasing environmental concerns have restricted the chemicals available for controlling this damage. As a result, biological control has received more interest. The application of these systems to wood has been limited due to their inability to reliably produce effective stain control under mill conditions. Many of these problems stem from a poor understanding of how these organisms function as bioprotectants.

In this thesis, a technique was identified to extract proteins from ponderosa pine sapwood samples colonized by bioprotectants and/or sapstain fungi. These extracts could be used to study the effects of the bioprotectants on various target stain fungi. The effects of extraction media and sample incubation period on protein recovery and enzyme activity in extracts were evaluated. Protein recovery and

activity in extracts were evaluated. Protein recovery and enzyme activity were affected by the extraction time, the medium conditions of the bioprotectants and the sample incubation conditions. The greatest protein recovery occurred, under the conditions of this study, when wood samples were incubated for thirty days and extracted for twelve hours using 50 mM sodium acetate buffer at pH 5.0 with 0.05 % Tween 80. The surfactant, Tween 80, greatly enhanced protein recovery. Longer incubation time (thirty days) was associated with greater produce cellobiase and xylosidase activities but, in contrast, glucosidase activities were greater with shorter incubation time (ten days), under the conditions of this study.

Extraction of Proteins from Wood Samples Colonized by Bioprotectants and/or Stain Fungi

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Li Cui, Author

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1 INTRODUCTION

Sapstain of wood (also called blue stain) is a common problem in both softwoods and hardwoods. Most sapstains are caused by fungi and bacteria. Stained wood is not accepted by customers and losses due to reduced marketability of the discolored wood have been conservatively estimated at about ten million dollars per year [Scheffer, 1973]. Stain losses, in fact, should be considerably greater than that figure because of the poor handling practices of many smaller sawmills and the hesitancy of sawmills to report losses. Stain losses generally increase with increasing sapwood content. Staining has become more noticeable because of an emphasis on natural finishes and the growth of markets for clear, light colored woods.

Biological discolorations caused by fungi and bacteria are typically controlled by either removing moisture from the freshly sawn wood as rapidly as possible or by the application of prophylactic biocides using dipping or spray systems [Zabel and Morrell, 1992]. However, in recent years, environmental concerns have led to increasing regulation of prophylactic fungicides. Alternative chemicals have been developed but few of them have been as successful as

pentachlorophenol, the chemical formerly used for this purpose [Drysdale, 1987]. Furthermore, recently formulated antistain fungicides may also have health and environmental effects.

An alternative to chemical antistain protection is biological protection (bioprotection) or biological control (biocontrol) which has received tremendous interest in recent years. A number of fungi and bacteria have been screened as potential biological protectants (bioprotectants) and some of them have shown the ability to inhibit fungal stain organisms. However, many of these tests were performed on artificial media under conditions that are quite different from those found in the field. Many potential bioprotectants have failed to perform well in the field. These failures reflect the lack of understanding of the modes of actions or mechanisms by which bioprotectants function on wood.

Bioprotectants are believed to function by competition, antibiosis and/or mycoparasitism [Mitchell, 1973]. Competition for space and nutrients may be the most common mechanism [Freitag et al.,1991]. Although the mechanisms by which bioprotectants function on other materials are well documented, little research has been performed on wood. One of the difficulties in assessing mechanisms is the absence of methods for reliably isolating potential antibiotics from wood incubated with bioprotectants and staining fungi.

In this study, a technique was developed to extract proteins from sapwood samples colonized by staining fungi and/or bioprotectants. The effects of extraction media, extraction time and sample incubation period on protein recovery and enzyme activity were evaluated to assess the effectiveness of the extraction procedures.

2. GENERAL INFORMATION ABOUT SAPSTAIN

Most species of sapstain fungi belong to the Ascomycetes and Fungi Imperfecti. Sapwood discoloration of timber is mainly due to the presence of pigmented hyphae [Zink and Fengel, 1988]. The coloring matter of sapstain fungi, melanin, is deposited in the hyphae walls or in the medium around fungal cells [Zink and Fengel, 1990]. The deposition of melanin begins at the earlier stages of hyphal growth and the melanization of the hyphae walls is connected with mycelial aging [Zink and Fengel, 1989].

Under favorable conditions, spores of stain fungi are airborne or carried by insect vectors to the surface of freshly sawn wood where they germinate within hours of landing and penetrate the wood surface through ruptured tracheids and exposed wood rays. The hyphae pass from one cell to another either by growing through pits or by direct penetration of the wood cell wall. Some species of blue stain fungi can even penetrate the lignified cell wall of tracheids by making small canals [Liese, 1970]. Pigmented hypha produce both aesthetic and physical changes in the wood. Early work on this subject was done by Scheffer and Lindgren [1940]. They studied the effects of stain fungi on bending and compressive strengths and noted that toughness (shock

resistance) of heavily stained softwood was significantly lower than that of normal wood. Stained wood consumes more bleach during pulping, thereby, increasing paper-production costs and stained wood is generally not recommended for structural purposes where strength is critical such as utility poles, laminated timbers, ladders or pilings. Additionally, stained wood has greater permeability and hygroscopicity due to the removal of pit membranes and can be wetted or dried more rapidly, increasing the risk of fungal decay [Zabel and Morrell, 1992].

3. SAPSTAIN CONTROL

Typically, sapstain control can be accomplished either through rapidly drying to remove moisture from wood or application of biocide solutions to protect the surface of timber against fungal invasion. These two approaches are called physical and chemical sapstain control, respectively.

3.1 Physical sapstain control

Moisture content of wood is very important for fungal or bacterial growth. Generally, if the moisture content of the wood is below 20%, the growth of most microorganisms becomes very limited [Panshin and de Zeeuw, 1980]. The most effective way to prevent stain and decay of timber is to rapidly reduce the moisture content of the wood below the fiber saturation point (generally about 30% moisture content) before the spores can germinate. In sawmills, the methods to reduce moisture contents in their products include kiln drying or air-seasoning. Many sawmills kiln dry their products to reduce the moisture contents below 19% before shipping. It subtantially reduces the possibility of losses from blue stain.

Drying not only protects lumber from stain or decay, but also provides dimensional stability, eliminating degrades caused by warping, splitting and checking of lumber, and minimizing shipping and handling costs [Brown, 1995]. However, kiln drying is very energy-intensive, may cause air pollution and does not limit re-invasion should wood be re-wetted.

3.2 Chemical control of sapstain

Chemical treatments for preventing stains have been employed for decades. Traditionally, chemical control of wood from sapstain can be accomplished by dipping or spraying the lumber with biocide solutions to protect the surface of the wood [Zabel and Morrell, 1992]. Dipping is relatively inexpensive. Where staining conditions are severe, both chemical protection and physical protection such as kiln dry are required.

Recently, however, potentially hazardous health and environmental effects as well as serious doubts regarding the safety of many chemical preservatives have led to the re-evaluation of formulations and closer scruity of industrial treatment practices [Greaves,1987]. Although pentachlorophenol continues to be widely used in some countries, increasing concerns over worker exposure, the

presence of dioxin contaminants, and the banning of this chemical in many countries have sharply diminished the use of penta for stain protection and resulted in an extensive evaluation to identify acceptable alternatives.

A number of alternative chemicals have been developed in the past ten years, but none of them has been as effective as pentachlorophenol [Miller et al., 1989]. Furthermore, these alternative chemicals may also have health and environmental problems [Drysdale, 1987]. Concerns about chemical safety have stimulated a search for alternative stain prevention strategies. One of the most intriguing strategies is biological protection (Bioprotection) or biological control (Biocontrol) of wood from stain or decay.

4. BIOLOGICAL CONTROL OF SAPSTAIN

4.1 The concepts of biocontrol of sapstain

The term, biological control, was first used in relation to plant pathogens by C.F. Von Tubeuf in 1914. H.S. Smith used the term biological control in relation to insects in 1919. Biological control or biological protection, in agriculture, is defined as:

> The decrease of inoculum or the disease-producing activity of a pathogen accomplished through one or more organisms, including the host plant but excluding man [Baker, 1987].

Biological control has received tremendous interest in agriculture and numerous successful agricultural applications of biological control of plant pathogens have been devised.

In wood or wood products, biological control has been desided by Freitag and Morrell as:

> One approach to non-chemical or integrated wood preservation employs antagonistic organisms which inhibit the growth of microorganisms causing biological deterioration by reducing their inoculum potential and their ability to invade wood and cause significant damage [Freitag et al. 1991].

It uses one or more organisms to inhibit the growth or damage effects of the target organisms [Kreber and Morrell, 1993]. Biological control in forest products has been studied primarily for its ability to prevent fungal degradation of wood [Ricard, 1966; Ricard and Bollen, 1968; Bruce and King, 1983, 1986a,b; Bruce and Highley. 1990,1991] or to eliminate incipient decay [Ricard et al., 1969; Morris and Dickinson, 1981; Highley, 1989]. Research on biological control against wood discoloration has received less attention, although the short period required for protection of wood from stain seems to have the highest potential for successful application of biological control.

The use of bacteria and fungi or other antimicrobial agents produced in a bioprotectant culture fluid to protect unseasoned lumber from biological discoloration has been the subject of considerable interest. A number of bacteria and fungi have been shown to inhibit fungal stain organisms in both artificial media and on wood samples. However, biological control agents often fail against certain fungi or under specific environmental conditions [Morrell and Sexton, 1992]. These failures may be due to the inability of the biological control agents to effectively colonize the substrate; to provide long term protection; or to inhibit the wide range of organisms which can damage wood. The inability

of biocontrol organisms to perform consistently under the variety of conditions to which the wood is exposed reflects an absence of understanding of the mechanisms by which biological control agents function on wood. This lack of the understanding not only limits the development of appropriate screening methods but also makes it impossible to enhance the biocontrol abilities. Better information on biocontrol mechanisms can be exploited to enhance important antagonistic traits or growth and survival capabilities, to make the organism more ecologically compatible with its target organism.

4.2 Proposed mechanisms of biological control

The mechanisms by which biological control agents function include competition, antibiosis and/or mycoparasitism [Freitag *et al*. 1991; Mitchell, 1973]. Competition for space and nutrients may be the most common mechanism and plays an important role in successful biological protection of wood from stain. Competition, as a mechanism, is also the most difficult mechanism to demonstrate.

Competition occurs when two or more organisms require the same material, whose use reduces the amount available to the other. Thus, microorganisms may compete for the same

nutrients: one organism sequesters more of the nutrients and grows because of better uptake mechanisms or more active extracellular enzymes, while another has insufficient nutrients and is inhibited. As heterotrophs, fungi and most bacteria are unable to manufacture their own food and utilize starches, cellulose, lignin and other nutrients in wood substrates for growth. Some microorganisms have been evaluated as potential bioprotection agents due to their higher growth rates which permit rapid exploitation of the substrate to exclude competitors. Many microorganisms exhibit poor abilities to completely colonize nutrient poor substrates such as wood [Seifert et al., 1987; Benko, 1988]. When a microbial population produces substances that are inhibitory or lethal to another population, the resulting mechanism is called 'antibiosis' and the substances produced are 'antibiotics' [Freitag *et al.*, 1991]. The organism that produces antibiotics may gain advantages by this mechanism or remain unaffected. Antibiosis may be a significant mechanism by which biocontrol of fungal stain occurs either through secretion of volatile or water soluble compounds that are either constitutively or inductively produced [Bruce and Highley, 1991b; Bruce et al., 1984]. Although some microfungi and bacteria produce antibiotics in liquid or solid media under high nutrient regimes, there are debates concerning the role of antibiotics in substrate colonization. In situ

production of antibiotics by biological protectants against stain fungi remains to be demonstrated. Furthermore, the antifungal compounds produced in culture fluid may not be uniformly inhibitory to all mold and stain fungi.

Mycoparasitism may be necrotrophic (destructive) or biotrophic (balanced). Necrotrophic parasites are defined as those that contact their hosts, excrete toxic substances that kill the host cells, and utilize the nutrients thus released. Biotrophic parasites are capable of obtaining nutrients from their hosts with little or no apparent harm to their hosts [Barnett and Binder,1973]. Mycoparasitism has been demonstrated in biological control of wood from stain [Benko and Henningson, 1986; Croan and Highley, 1991a], however, the organisms employed may also discolor the wood due to mass production of colored spores on the wood surface. The importance of this mechanism in biological control of wood stain is uncertain, because the organisms to be parasitized have already colonized the wood and the purpose of biocontrol is to prevent such colonization.

Generally, an ideal wood biocontrol agent should be able to rapidly colonize and occupy a vacant resource such as wood, utilize the nutrients available on the surface of substrate (wood) or from storage cells within the wood and change the

substrate sufficiently to prevent colonization by other competing microorganisms such as decay and stain fungi without harming the appearance and structural integrity of the wood. Additionally, an ideal biological control agent should provide long-term protection without safety and environmental problems.

5. LITERATURE REVIEW OF BIOLOGICAL CONTROL OF SAPSTAIN

The first observation that blue stain in softwood was caused by a fungus possessing dark-brown hyphae was made by Hartig in 1878 who identified a fungus then known as *Ceratostomella piliferum*. For a long time, *C. piliferum* was looked upon as the specific fungus responsible for the bluing of coniferous timber. In 1906, Hedgcock described a number of new species capable of causing stain in pine and sweetgum [Findlay, 1959]. Extensive studies on blue stain made by Scheffer and Lindgren (1940) in the 1930's showed that stain-prevention depended on either rapid processing to dry the wood below the fiber saturation point or surface application of prophylactic biocides to prevent microbial colonization. However, increasing concerns about the safety of the chemicals have stimulated the research to identify safer strategies. One of the alternatives is biological control.

Biological control of wood or forest products from sapstain and decay has received considerable recent interest. A number of researchers have evaluated the activities of bacteria and fungi against sapstain and decay fungi. While many trials have been performed on artificial media, these tests indicate that some bacteria and fungi can inhibit biological deterioration under certain conditions.

5.1 Screening of potential bioprotectants

Most biological control studies on wood have focused on screening of potential bioprotectants and most employed cross plating on agar or wood samples under laboratory conditions. These tests might ignore the sensitivity of bioprotectants to environmental factors under which they are exposed.

Bernier *et al* (1986) reported that *Bacillus subtilis* prevented colonization of *Picea* sp. blocks by three sapstain or mold fungi. Florence and Sharma (1990) also studied the ability of *B. subtilis* to inhibit the growth of *Botrydiplodia theobromae* Pat. on various important Indian wood species. Among these wood species, rubber wood (*Hevea brasiliensis*) blocks, one of the most decay susceptible wood species, was completely protected for two weeks after a five second dip in a suspension of *B. subtilis*.

Seifert *et al* (1987) evaluated a strain of *Bacillus subtilis* C186 as a potential biological control agent of sapstain and mold fungi on unseasoned lumber and briefly reviewed some of the applications of biological control of forest products for prevention of sapstain. They subsequently evaluated the antagonistic abilities of eighty-eight strains of fungi in agar culture and wood samples against sapstain fungi [Seifert

et al., 1988]. *Acremonium strictum* 314A, *Byssochlamys nivea* 681A, *Penicillium thomii* 655B and *Stibella aciculosa* 663A were identified as promising biological control organisms.

One hundred fifty bacteria belonging to fifteen genera were evaluated for their abilities to inhibit fungal growth on dual cultures [Benko, 1988]. *Pseudomonas cepacia* Burkholder exhibited strong antagnonistic activity against *A. pullulans*, *C. coerulescens* and *Ceratocystis* spp.. Later, *P. cepacia* isolate #6253 effectively limited sapstain over a four month period on *Pinus radiata* D. Don.[Benko, 1989]. Moisture gradients of logs under field conditions were quite different from those in the wood specimens under laboratory conditions. Field trials with *P. cepacia* produced complete protection for only three weeks [Benko, 1989].

Kreber and Morrell (1992) studied the potential of nine bacteria and six fungi to inhibit fungal stain of unseasoned ponderosa pine sapwood on small wood samples exposed in a moist environment. *Bacillus subtilis* Cohn isolate 733A, *Gliocladium vireas* J.H Miller, J-E. Giddens & A.A. Foster isolate H3 and *P. putida* Migula isolate A-12 were identified as promising biological agents. Performance of two *B. subtilis* isolates was closely related to the media on which

the organisms were grown prior to wood application. The best protection results were obtained when the isolates were incubated on their preferred medium. *Bacillus subtilis* 733A was the most effective one of the fifteen isolates tested, but there was no evidence of antibiotic production. It was suggested that a better understanding of microbial interactions on wood surface was essential for further development of this stain prevention strategy.

5.2 The antibiotic effects of bioprotectants on stain fungi

In the 1990's, a strain of *Streptomyces rimosus* was intensively studied at the USDA Forest Products Laboratory in Madison, Wisconsin. These studies focused on the ability of the metabolites from this bacterium to inhibit various sapstain fungi.

Investigations on antibiotics as possible microbial control agents on wood were begun in the 1960's. One of the earliest reports by Klingstrom and Beyer showed that some *Scytalidium* spp. inhibited the growth of the wood-rotting fungi, *Fomes annosus* (FR.) [Klingstrom and Beyer, 1965] and *Poria carbonica* Overh [Ricard and Bollen, 1968]. Later, an antibiotic named scytalidin extracted from cultures of *Scytalidium* was shown by agar plate toxicity tests to inhibit

a wide spectrum of microorganisms including wood decay and staining fungi [Stillwell *et al.*, 1973]. Similarly, other antibiotics from some *Hyalodendron* spp. were found to inhibit wood-colonizing microorganisms including staining fungi [Stillwell *et al.*, 1969]. Stranks also noted that these extracted antibiotics were capable of inhibiting blue stain fungi in pine sapwood.

Highley *et al.* (1991) obtained excellent control of sapwood-inhabiting fungi in wood dipped into preparations of several bacteria, including *Streptomyces rimosus*. *Streptomyces rimosus* was the most effective of the bacteria tested, giving complete protection against discoloration. This bacterium alone was even slightly more effective than a mixture of the bacteria tested. The results indicated that the metabolites produced by *S. rimosus* were the most effective of all the bacteria tested for controlling sapstain and mold fungi. Benko and Highley (1990b) suggested that the inhibition was due to the synthesis of antifungal compounds.

In a study of antifungal activity of metabolites from *S. rimosus*, Croan and Highley (1991b) evaluated the efficacy of antifungal metabolites from *S. rimosus* for controlling the growth of sapstain fungi *C. coerulescens*, *C. minor*, *C.*

pilifera, *A. pullulans* and mold fungi *Aspergillus niger*, *Penicillium* spp. and *Trichoderma* spp. by using petri plate, wood blocks and log sections. Metabolites of this bacterium inhibited the mycelial growth of these sapwood-inhabiting fungi in both petri plate assays and plate bioassays. Concentrated metabolites (10X) from *S. rimosus* effectively controlled the growth of sapwood-inhabiting fungi on wood blocks under laboratory conditions. Inhibition was attributed to the production of antifungal compounds by the bacterial bioprotectants evaluated [Croan and Highley, 1991b].

Inhibition of spore germination of mold and stain fungi by unconcentrated metabolites from *S. rimosus* with boron as a co-biocide was studied by Croan and Highley (1992). Boron acted synergistically to protect wood from mold and stain attack. The protection was better than that obtained with concentrated metabolites (10X) [Croan and Highley, 1992]. Similar results were also obtained by Dawson-Andoh and Morrell using fluoride and boron with *Trichoderma harzianum*(1990). Live *S. rimosus* was highly effective against sapwood-inhabiting fungi on southern pine and sweetgum wood blocks under laboratory conditions and in the field exposure trials with green pine log sections [Croan and Highley, 1992]. Live bacterial cells completely protected green pine log sections from sapwood-inhabiting fungi such as *C.*

coerulescens, *C. minor*, *C. pilifera* and *A. niger*. *Streptomyces rimosus* is a non-wood-decay organism that does not alter the physical state of wood. Strains of this bacterium produce antibiotics such as oxytetracycline, tetracycline and the antifungal agent rimocidin. Production of antifungal metabolites is advantageous to a biocontrol system because the effect may remain in wood after the biological control organisms die or the antifungal metabolites could be used to treat wood.

5.3 The effects of mycoparasitism on staining

In 1986, over one hundred fungi were evaluated for their abilities to inhibit *Ceretocytis coerulescens* Bakshi using malt agar cross platings [Benko, 1986]. Among those fungi, some from *Gliocladium* spp., *Trichoderma* spp. and two mycorrhizal fungi *Clitocybe geotropa* Bull Quel. and an unidentified strain named D37 showed strong antagonistic ability. Benko (1987) subsequently demonstrated that extracts of fungal cultures of two mycorrhizal fungi were more active against *C. coeruleens* in a malt agar cross plating test and on pine (*Pinus sylvestris* L.) specimens.

Benko and Henningsson (1986) also investigated the abilities of twenty-two mycoparasitic fungi against *C. coerulescens*, *Aurobasidium pullulans* [deBary] Arnaud and *Sclerophoma* spp. They noted that the white rot fungus *Bjerkandera adjusta* [Willd.: Fr.] Karst was highly parasitic and penetrated into the hyphae of the blue stain fungus. An almost complete bleaching of stain hyphae was observed and it was suggested that enzymatic reactions, probably involving peroxidases, might be responsible for this effect [Benko and Henningsson, 1986]. This finding might imply another approach for controlling sapstain by biological means.

5.4 Studies on competition of bioprotectants

5.4.1 The effects of pre-treatment on the competitive abilities of some bioprotectants

A critical aspect of biological control is to make the bioprotectants rapidly and uniformly colonize the wood. Wood contains low levels of nutrients required for the growth of many potential bioprotectants. Dawson-Andoh and Morrell (1990) examined the abilities of fluoride and boron to stimulate growth and colonization by *Trichoderma harzianum* Rifai (#25) against *Postia placenta* (Fr.) M. Lars. et Lomb. (Madison R-105) in small wood wafers. Application of

relatively low levels of diffusible chemicals such as boron or fluoride can alter the wood environment to favor the bioprotectants.

Morrell and Sexton (1990) explored the feasibility of using volatile chemicals such as Vapam[R] and chloropicrin to encourage the growth of biological control agents *T. harzianum*, *T. polysporum* and *S. aurantiacum* in a " Sandwich" test. Wood moisture content varied with positions of the colonized wafers and affected the ability of the biological control agents to inhibit *P. placenta* and Lomb., *Irpex lacteus* Fr., and *Tramates versicolor* (L.: Fr.) Pilat.

5.4.2 Effects of environmental and media factors on the competitive abilities of some bioprotectants

The poor field performance of potential bioprotectants due to sensitivity to environmental factors such as temperature, nutrient, wood species or the presence of competing microflora has stimulated a search for methods for encouraging bioprotectant performance. Morrell and Sexton (1992) examined the effects of nutrient regimes, incubation temperature, media pH, and wood sterilization method on the performance of *Pseudomonas putida*, *P. cepacia*, *Bacillus subtilis* and *Trichoderma harzianum* using small Ponderosa pine samples over a four week period. Bioprotectant performance

was affected by a variety of nutritional, environmental and wood conditions, making it difficult to achieve complete protection against fungal stain under the wide array of conditions which characterize freshly sawn wood. Successful bioprotection requires identification of organisms capable of competing with the resident microflora on the wood under various conditions. It may also be possible to manipulate the components of the system to enhance bioprotectant efficacy.

Benko and Highley (1990a) noted the effect of media composition on the antagonism of six bacteria including *P. cepacia* to four wood-attacking fungi including *C. coerulescens* and *T. harzianum* on twenty-two different media. Fungi grew poorly on bacterial media suggesting that components in bacterial media may have some use for selectively encouraging bacterial growth. The most suitable media demonstrating antagonistic effects were yeast dextrose peptone agar and sporulation agar [Benko and Highley, 1990a]. In a similar study the same authors grew the same six bacteria in fourteen different media and treated wood samples with a mixture of these bacteria before exposing the wood to four wood-attacking fungi. The degree of the surface staining

served as the indicator of bioprotection. The bacterial preparation grown on sporulation broth gave the best protection against the fungi tested [Benko and Highley, 1990b].

There is a significant need for a better understanding of the mechanisms by which bioprotectants function, to effectively identify metabolites in situ and to establish the effects of these compounds on the target organisms will provide an important tool for achieving the goals. The development of effective methods for extracting metabolites and enzymes from wood colonized by both bioprotectants and stain fungi.

6. OBJECTIVES

The objectives of this study were:

1. To identify a technique to extract proteins from sapwood samples colonized by bioprotectants and/or stain fungi.

2. To evaluate the effects of extraction media, time and incubation time on protein recovery and enzyme activity of bioprotectants and stain fungi in wood.

3. To study the effects of the selected bacterial bioprotectants on wood stain fungi.

7.MATERIALS METHODS

7.1. Wood Samples:

Defect free samples (5x10x30mm) were cut from Ponderosa pine sapwood (*Pinus ponderosa* Laws) which had not received prior chemical treatment. Ponderosa pine has low resistance to stain and its sapwood is easily separated from heartwood due to the marked color difference. These samples were vacuum soaked with water and sterilized by exposure to 2.5 mrads of gamma radiation from a cobalt 60 source. Irradiation minimized the possible changes in wood components that are associated with the use of heat sterilization [Morrell and Sexton, 1992].

7.2. Preparation of bioprotectant inoculum

7.2.1 Organisms:

Bacillus subtilis Cohn (Isolate BS 733A, Forintek Canada Corp, Ottawa, Canada) and *Pseudomonas fluorescens* Migula (Isolate Pf-5, J.E. Loper, USDA, ARS, Corvallis, OR) were chosen as test bioprotectants. These two organisms have shown

activity against stain fungi in prior trials [Kreber,1991; Benko,1988,1989].

7.2.2. **Selection of Media Conditions for Bioprotectant Systems:**

Bioprotectants were grown on nutrient broth at pH 9.6 at room temperature on a rotary shaker for 4 to 6 days prior to the application on wood. The pH of the medium was controlled by addition of glycine-NaOH buffer. Generally, most bacteria have more alkaline pH optima while most fungi prefer more acidic conditions [Morrell and Sexton, 1992] . Coniferous wood is generally acidic (pH 3 to 5) [Cowling and Merrill,1966].

7.2.3 Preparation of bioprotectant inoculum:

A small plug (4x4 mm) of bioprotectant inoculum from the stock culture was transferred to a 250 ml Erlenmeyer flask containing 75 ml of the selected media. Each bioprotectant was inoculated into four flasks which were incubated at room temperature (23-25°C) for 4-6 days prior to use.

7.3. Preparation of stain fungi inoculum:

7.3.1 Organisms:

The stain fungi employed in this study are listed in table 7.3.1 with their current names and strain numbers. The stain fungi were prepared as a spore/hyphal fragment suspension prior to application. Along with the stain fungi, *Aspergillus niger* was included since it is a fast-growing mold fungus.

7.3.2 Preparation of fungal stain inoculum:

A small agar plug cut from the stock culture of a given fungus was inoculated into a 250 ml Erlenmeyer flask containing 50 ml of 1.0% malt-extract broth. Each fungus was inoculated in one flask. The flasks were incubated at room temperature (23-25°C) until abundant dark pigmented mycelium developed. The contents of the flasks were vacuum filtered through Whatman #4 filter paper. Spores and hyphal fragments were washed off the filter paper into a 500 ml beaker with sterile distilled water and blended for 15 seconds. Sterile

distilled water was added to the blended biomass to obtain 250 ml of a fungal spore/hyphal fragment mixture. The suspensions for each of the four fungi were combined in a squeeze bottle for wood application.

Table 7.3.1: Mold and stain fungi employed to study bioprotectant capabilities.

SPECIES	STRAIN NUMBER	SOURCE
Alternaria alternata (FR:FR) Keissl.	ED 113	CJK WANG Syracuse
Cladosporium elatum Nann	S63-1-1	FRL,OSU
Phialophora fastigiata Conant	14	CJK WANG Syracuse
Aspergillus niger van Tieghe	ATCC9642	ATCC

7.4. Treatment of wood samples:

7.4.1 Treatment of wood samples:

The sterilized wood samples were dipped for 30 seconds in the appropriate media with or without bioprotectants, then allowed to drain and surface dry for 1-2 minutes. The samples were then sprayed to runoff on both sides with the prepared stain fungal suspension. Additional samples were inoculated

with sterile distilled water alone, stain fungi alone and bioprotectant alone to serve as controls. Each treatment combination was replicated on twenty-four samples in three petri plates (eight/per plate).

7.4.2 Incubation periods and temperature

The samples, including controls, were placed in glass petri plates on U-shaped glass rods over two moistened #3 Whatman filter papers. Petri plates were incubated at room temperature (23-25° C) for ten or thirty days.

7.5.Extraction of wood samples:

7.5.1 Extraction media:

The extraction media employed were:

A: 50 mM sodium acetate buffer at pH 5.0.

B: 50 Mm sodium acetate buffer at pH 5.0 with 0.05 % Tween 80.

C: Sterile distilled water at pH 7.0.

7.5.2 Extraction periods and temperature:

All extractions were performed at room temperature (23-25° C) for three, six or twelve hours.

7.5.3 Procedures:

The wood samples were harvested after incubating ten or thirty days. Twenty-four wafers from each treatment were placed in a 250 ml flask, and 50 ml of the appropriate extraction media was added. These flasks were placed on a rotary shaker (100 rpm.) at room temperature(23-25° C) for 3, 6 or 12 hours. At each selected time point, one-third of the flasks from each treatment combination were removed from the shaker and the contents were filtered through Whatman #4 filter paper, followed by 0.4 um and 0.24 um membrane filters. The filtrate was then concentrated from 50 ml to 10 ml using a Stirred Ultra-Filtration Cell with an Amicon Diaflo YM10 membrane with a molecular weight cutoff of 10,000. The higher molecular weight concentrates were redissolved in sodium acetate buffer and reconcentrated to remove dark pigments that might interfere with spectrophotometer readings. Controls were prepared in the

same way. The extracts were prepared for each treatment/medium/incubation time/extraction time combination. All treatments were evaluated in duplicate and stored at -20° C prior to analysis.

7.6. Protein and enzyme activity assays:

7.6.1 Total protein content:

Total protein content was determined using a Bio-Rad protein assay kit [1989 Bio-Rad, Richmond, CA]. The Bio-Rad protein assay is a simple and accurate procedure for determining the concentration of solubilized proteins [Braford, 1976; Spector 1979]. The Bio-Rad protein assay is a dye-binding assay in which color changes of the dye occur in response to various protein concentrations. For this test, bovine serum albumin served as a standard to quantify the amounts of protein in the extracts.

7.6.2 Assays of enzyme activity:

7.6.2.1 Buffer:

All enzyme assays were performed in 50 mM sodium acetate buffer at pH 5.0.

7.6.2.2 Substrates:

Each extract was evaluated for enzyme activity with particular emphasis on cellulase and hemicellulase enzymes which are common to the wood stain fungi.

Enzyme activity assays were based upon the release of p-nitrophenol from p-nitrophenol-sugar analogs [Desphande et al., 1988;Lachke, 1988;Michaeland Schmidt, 1988; McCleary, 1988]. The p-nitrophenol derivatives of β-D-glucopyranoside, β-D-cellobioside and β-D-xylopyranoside (Sigma Chem. Co., St.Louis, MO) were used as substrates in the enzyme assays.

7.6.3 Assay procedures:

Two hundred ul of wood extract was added to 800 ul of 50 mM p-nitrophenol sugar analog in 50 Mm sodium acetate buffer at pH 5.0 and incubated at 50°C for one hour. The reaction was

stopped by adding 4.0 ml of 0.4 M glycine buffer (pH 10.8). At alkaline pH, p-nitrophenol is in intense yellow color. The p-nitrophenol released from the p-nitrophenol sugar analogs was determined by measuring absorbance at 430 nm with p-nitrophenol as the standard. One unit of enzyme activity (nKatal) was defined as the amount of enzyme that released 1 nM of p-nitrophenol per minute. All assays were performed in duplicate on all extracts of each set of treated samples including controls.

7.6.4 Statistical Analysis

An analysis of variance(ANOVA) was employed to examine the differences between total protein and cellobiase, glucosidase or xylosidase activities from wood samples exposed to bioprotectants and/or sapstain fungi on different treatment combinations. A multiple range analysis procedure was used to test the differences between all means [Statgraphics 5, Statistical Graphics Corporation]. The results are shown in Appendix tables.

8.RESULTS AND DISCUSSION

8.1. Total Protein Levels

8.1.1. Effects of wood sample incubation time on protein recovery

Increasing exposure time of wood samples to the bacterial bioprotectants or stain fungi resulted in increased protein recovery. Protein levels recovered from wood samples incubated for ten days were generally lower than those from samples incubated for thirty days for the same treatment combination (Tab.1.1), although the highest protein levels were obtained from samples exposed to *Pseudomonas fluorescens* alone, incubated for ten days and extracted for six hours in 50 mM sodium acetate buffer at pH 5.0 with 0.05% Tween 80.

Generally, longer incubation times produced better protein recovery, although there were some variations. These trends suggest that while some protein is initially applied as part of the inoculum, the organisms continue to grow and synthesize cell wall materials.

Nutrients and space on the wood surface are very limited. The acidic pH of many coniferous woods (pH 3-5) [Cowling and Merrill, 1966] is in the optimum pH range for growth of many

stain fungi while bacteria generally require neutral or more basic pH's. Competition of fungi and bacteria for nutrients and space on the wood surface can initially stimulate metabolic activity and protein production. Eventually, however, these organisms utilize the ready available nutrients and the growth rate declines, bringing concurrent reductions in levels of enzymes and other proteins.

8.1.2. Effect of extraction time on protein recovery

Total protein levels from wood samples incubated for ten days and extracted for six hours were slightly higher than those extracted for three or twelve hours. Protein levels increased as extraction time increased in the same treatments for samples incubated for thirty days and the highest protein levels were produced from wood samples extracted for twelve hours. Statistical analysis also indicated that total protein contents from wood samples extracted for three hours were significantly lower than those extracted for twelve hours when the wood samples were incubated for thirty days (Appendix 2).

Table 8.1.1: Total protein recovery from ponderosa pine samples exposed to combinations of bioprotectants and stain fungi for 10 or 30 days[a].

Treatment			Extraction Media		Protein Recovery(mg/ml) Extraction Time					
					Three Hrs		Six Hrs		Twelve Hrs	
Stain Fungi	Biop		Buf.	TW80	Incubation Period		Incubation Period		Incubation Period	
	B	P			10 Days	30 Days	10 Days	30 Days	10 Days	30 Days
-	-	-	-	-	0.33	0.68	0.47	0.28	0.16	3.05
-	-	-	+	-	0.12	0.83	0.21	1.3	0.24	0.26
-	-	-	+	+	11.62	3.295	15.69	7.278	10.29	0.26
+	-	-	-	-	0.32	0.58	0.46	0.58	0	13.71
+	-	-	+	-	0.06	0.56	0.9	1.09	0.07	0.45
+	-	-	+	+	3.48	7.57	5.86	1.57	4.02	9.18
-	-	+	-	-	1.67	2.25	2.18	6.28	1.87	2.96
-	-	+	+	-	5.77	1.66	2.61	3.77	6.72	1.95
-	-	+	+	+	15.3	6.1	13.0	7.73	12.7	10.02
+	-	+	-	-	0.47	0.01	0.27	3.29	8.14	1.67
+	-	+	+	-	1.30	4.84	2.18	2.35	1.14	5.53
+	-	+	+	+	7.56	4.28	8.10	4.30	5.70	8.69
-	+	-	-	-	0.64	0.19	1.46	1.48	0.85	2.78
-	+	-	+	-	0.42	1.15	0.81	1.87	0.74	6.48
-	+	-	+	+	8.05	4.94	6.71	5.81	9.09	6.66
+	+	-	-	-	2.17	3.34	0.61	6.21	1.12	6.47
+	+	-	+	-	1.36	2.00	1.72	3.78	1.44	5.53
+	+	-	+	+	8.50	4.23	10.93	4.73	8.49	8.51

a. where B: *Bacillus subtilis* and P: *Pseudomonas fluorescens*. Samples were extracted in solution with or without 50 mM sodium acetate buffer at pH 5 and Tween 80.

The microbial extracellular proteins from the organisms on wood surface are produced at very high levels shortly after inoculation as organisms compete for nutrients and space. As nutrients and space become limiting and microbial growth rates reach their peak, and then decline. Eventually, some extracellular proteins may be autolyzed and the production of proteins may decline. In this study, however, the production of proteins increased as the exposure time increased, suggesting that active microbial systems were still present in the wood.

8.1.3 Effect of extraction media and pH on protein recovery

Protein levels recovered from wood samples extracted using 50 mM sodium acetate buffer at pH 5.0 with 0.05% Tween 80 were significantly higher than those extracted with distilled water or 50 mM acetate buffer without Tween 80(Tab.1.3). Microbial extracellular proteins can be bound or adsorbed to membranes and the wood, and may also be aggregated within the wood cell-lumens due to their hydrophobic nature [Errson et al. 1989]. Tween 80 is a non-denaturing and nonion surfactant

which decreases the hydrophobic interactions between proteins and wood, increasing solubility of membrane-bound protein and improving protein recovery.

Protein solubility is affected by many factors including pH and ionic strength. Interestingly, there was no significant difference between the protein contents of wood samples extracted with distilled water at pH 7.0 or 50 mM sodium acetate buffer at pH 5.0 without Tween 80. Application of the surfactant, Tween 80, further enhanced protein recovery. Previous studies have shown that including 0.05 % Tween 80 in 50 mM sodium acetate buffer at pH 5.0 substantially improved protein recovery [Dawson-Andoh and Morrell, 1992].

Table 8.1.2: Effect of extraction media on total protein recovery from ponderosa samples exposed to combination of bioprotectants and stain fungi [a].

Treatment			Extraction Time (Hrs)			Protein Recovery (mg/ml) Incubation Period					
						10 Days			30 Days		
SF	Biop		3	6	12	Extraction Media					
	B	P				W	Buf.	BT	W	Buf.	BT
_	_	_	+	_	_	0.32	0.12	11.62	0.68	0.83	3.30
_	_	_	_	+	_	0.47	0.21	15.69	0.28	1.30	7.28
_	_	_	_	_	+	0.16	0.24	10.28	3.05	0.26	0.26
+	_	_	+	_	_	0.32	0.06	3.40	0.58	0.56	7.57
+	_	_	_	+	_	0.46	0.90	5.86	0.58	1.09	1.57
+	_	_	_	_	+	0	0.07	4.02	13.71	0.45	9.18
_	_	+	+	_	_	1.67	5.77	15.30	2.24	1.66	6.10
_	_	+	_	+	_	13.00	2.61	2.18	6.29	3.77	7.73
_	_	+	_	_	+	1.87	6.72	12.73	2.96	1.95	10.02
+	_	+	+	_	_	0.47	1.50	7.56	0.01	4.04	4.30
+	_	+	_	+	_	8.10	2.18	0.27	3.29	2.35	4.30
+	_	+	_	_	+	8.14	1.14	5.70	1.67	5.53	8.69
_	+	_	+	_	+	0.64	0.42	0.05	0.19	1.15	4.94
_	+	_	_	+	_	1.46	0.81	6.71	1.48	1.87	5.81
_	+	_	_	_	+	0.85	0.74	9.09	2.78	6.47	6.66
+	+	_	+	_	_	2.17	1.35	0.50	3.34	2.00	4.23
+	+	_	_	+	_	0.61	1.72	10.93	6.21	3.78	4.73
+	+	_	_	_	+	1.12	1.44	8.49	6.47	5.53	8.51

a. where B: *B. subtilis,* P: *P.fluorescens* and SF: stain fungi. Biop. bioprotectants. Extraction media included (W) Water, (Buf.)50 mM sodium acetate buffer and (BT) 50 mM sodium buffer with 0.05% Tween80.

8.2. Enzyme activities

8.2.1. Cellobiase activity

Cellulose represents 40 to 50 % of the lignocellulose matrix of wood. Cellulose can be decomposed by a complex system involving at least three different enzymes through a series of hydrolytic reactions. Cellobioses are produced during the breakdown of cellulose and are the intermediate products in degradation of cellulose to glucose. Cellobiase hydrolyzes cellobiose from the crystalline portion of the cellulose.

Cellobiase activity varied somewhat among the various treatment combinations. The absence of cellobiase activity may reflects the activity of the bioprotectant but cellobiase activity was also absent in a number of treatments with stain fungi alone. This absence of activity may reflect losses in activity due to methodology. Activities tended to be higher where samples were incubated for thirty days. For most treatment combinations, cellobiase activity was significantly higher when samples were extracted for three or six hours. The cellobiase activity levels (CAL) from the extracts extracted for three, six or twelve hours could be statistically ordered as: $CAL_{3h}>CAL_{6h}>CAL_{12h}$, no matter how long the wood samples were incubated (Appendix 5 & 6). The

effect of extraction media on cellobiase activity was varied and Tween 80 did not appear to improve cellobiase activity in the extracts in this experiment.

Neither *Bacillus subtilis* nor *Pseudomonas fluorescens* exhibited significant cellobiase activity in extracts from samples exposed to these organisms alone, reflecting their inability to metabolize cellulose on wood under the conditions of this experiment. This absence of substantial wood effects is an important characteristic of successful bioprotectants.

In general, cellobiase activity decreased as extraction time increased, suggesting that this enzyme might be sensitive to protease or other degrading agents present in the extraction media. The highest cellobiase activities were obtained from wood samples exposed to both the bioprotectants and stain fungi, incubated for ten days and extracted for three hours. The results also indicated that *B. subtilis* 733A and *P. fluorescens* Pf-5 did not completely inhibit cellobiase activity of the selected stain fungi in this experiment.

Table 8.2.1: Effect of incubation period in the presence of bioprotectants and stain fungi and subsequent extraction time on cellobiase activity[a].

Treatment			Extraction Media		Cellobiase Activity (nKatals)					
					Incubation Period					
					10 Days			30 Days		
Sf	Biop.		Buf.	TW80	Extraction Time			Extraction Time		
	B	P			3 Hrs	6 Hrs	12 Hrs	3 Hrs	6 Hrs	12 Hrs
–	–	–	–	–	0	0	0	0	0	0
–	–	–	+	–	0	0.10	0	0	0.51	0
–	–	–	+	+	0.08	0	0	0	0	0
+	–	–	–	–	0	0	0	0	0	0
+	–	–	+	–	0	0	0	0.01	0	0
+	–	–	+	+	0	0	0	0.22	0.02	0
–	–	+	–	–	0	0	0	6.84	2.42	0.44
–	–	+	+	–	0	0	0	4.89	0.03	0.03
–	–	+	+	+	0	0	0	2.89	0.72	0
+	–	+	–	–	0.78	0	8.13	5.32	1.83	0
+	–	+	+	–	2.58	2.23	0.73	4.19	0.85	0
+	–	+	+	+	3.60	2.00	1.08	5.68	1.65	8
–	+	–	–	–	0.45	0	0.84	0.49	0.84	0.10
–	+	–	+	–	0.10	0.11	0.12	3.59	0.42	0
–	+	–	+	+	0	0	0.01	4.77	1.94	0
+	+	–	–	–	4.14	1.84	0.37	4.42	8.29	0
+	+	–	+	–	1.47	0.68	2.81	4.64	3.63	0
+	+	–	+	+	3.56	2.49	1.98	1.79	4.67	0

a. where, B: *B. subtilis*, P: *P. fluorescens*,
Buf.: 50 mM sodium acetate buffer at pH 5.0
Sf: Stain fungi. TW80: Tween 80.

8.2.2 Glucosidase activity

Glucosidase is an important component of the cellulase system releasing glucose from cellobiose. Glucosidase activity was not detected in control samples extracted in water or sodium acetate buffer without Tween 80. The presence of Tween 80 resulted in detection of glucosidase activity in these extracts. The reasons for this false positive are unclear. Glucosidase activity was highest in extracts from samples extracted for three hours, followed by twelve and six hours, respectively (Tab. 2.2.), but the results did not differ statistically nor did extraction media significantly alter glucosidase activity(Appendix 9 & 10).

Glucosidase activity was consistently higher in wood samples exposed to both *B. subtilis* and stain fungi. Glucosidase activity was lower in samples exposed to both *P. fluorescens* and stain fungi, suggesting that this bacterium was more active against the stain fungi. Glucosidase activity levels generally declined in samples incubated for thirty days, suggesting that fungal activity had declined over that period. Extraction efficiency appeared slightly better at twelve hours although there were inconsistencies in these results.

Table 8.2.2: Effect of incubation period, in the presence of bioprotectants and stain fungi, and extraction time on glucosidase activity of ponderosa pine samples[a].

Treatment			Extraction Media		Incubation Period					
					10 Days			30 Days		
Sf	Biop.		Buf.	TW80	Extraction Time			Extraction Time		
	B	P			3 Hrs	6 Hrs	12 Hrs	3 Hrs	6 Hrs	12 Hrs
–	–	–	–	–	0	0	0	0	0	0
–	–	–	+	–	0	0	0	0	0	0.02
–	–	–	+	+	0	0	0	0	0	0.01
+	–	–	–	–	0.06	0	0	0.06	0	0
+	–	–	+	–	0.02	0.17	0.67	0	0.04	0
+	–	–	+	+	0.05	0.19	0.02	0.04	0	0
–	–	+	–	–	0	0.08	0	0.38	0	1.38
–	–	+	+	–	0	0	0	1.16	0	1.10
–	–	+	+	+	0	0	0.01	1.22	0.02	2.06
+	–	+	–	–	0.41	0	0.92	0.92	0.42	0.31
+	–	+	+	–	2.63	3.80	2.45	1.57	0.26	0.07
+	–	+	+	+	2.04	0.44	3.18	1.36	0.61	8
–	+	–	–	–	0	0.21	0.45	0.03	0.03	2.65
–	+	–	+	–	1.00	0.92	1.21	0.37	0.07	0.34
–	+	–	+	+	0.35	0.06	0.51	0.72	0.33	1.06
+	+	–	–	–	5.07	3.06	2.15	0.67	1.58	0.08
+	+	–	+	–	4.08	3.42	3.63	0.97	1.04	0
+	+	–	+	+	5.07	3.35	3.59	1.68	0.73	0.05

a: where B: *B. subtilis*, P: *Pseudomonas fluorescens*,
Sf: Stain fungi. Buf.: 50 mM sodium acetate buffer at pH 5.0
TW80: Tween 80.

8.2.3. Xylosidase Activity

Hemicelluloses constitute 20 to 35 percent of the total dry weight of wood. These polymers have shorter chains compared to cellulose and are generally more susceptible to microbial attack. Xylans are important components of hemicelluloses that are formed by the polymerization of the anhydrous forms of xylose and other sugar molecules. Xylan decomposition requires the action of several xylanolytic enzymes. Xylosidase hydrolyzes xylan fragments or the xylo-oligosaccharides to xylose.

Xylosidase activity varied widely among the various treatment combinations. Activity was generally absent when stain fungi were not present or when one of the bioprotectants was present(Table 2.3). Xylosidase activity was absent in extracts from samples exposed to sterile distilled water alone, stain fungi alone or to *P. fluorescens* alone regardless of extraction time. Previous studies on the effects of sapstain on physical properties of wood indicate that bending and compressive strengths are not seriously affected by sapstain but toughness or shock resistance of heavily stained softwood may be much lower than that of normal wood [Scheffer and Lindgren, 1940]. This loss may reflect the disruption of the lignocellulasic matrix through

hemicellulose removal. The absence of substantial xylosidase activity in samples exposed to stain fungi is perplexing, but it may reflect the timing of extraction. Hemicellulose is typically viewed as the first polymer utilized by most fungi. It is possible, however, that the stain fungi rapidly utilized accessible hemicelluloses prior to the first sampling point, then the levels of this enzyme subsequently declined. Activities after thirty days of incubation also varied, although levels were typically highest when a three hour extraction time was used regardless of extraction media.

Xylosidase activity from wood samples exposed to both bioprotectants and stain fungi decreased as extraction time increased. The highest levels of xylosidase activity were obtained from wood samples extracted for three hours and exposed to both *B. subtilis* and stain fungi (Tab. 2.3). Statistical analysis indicated that the order of xylosidase activity levels (XAL) from extracts with different extraction times was: $XAL_{3h}>XAL_{6h}>XAL_{12h}$ and there were no significant differences in xylosidase activity in extracts prepared using the three extraction media.

Table 8.2.3: Effect of incubation period in the presence of bioprotectants or stain fungi and extraction time on xylosidase activity of ponderosa pine samples[a].

Treatment			Extraction Media		Xylosidase Activity(nKatals)					
					Incubation Period					
					10 Days			30 Days		
Sf	Biop.		Buf.	TW80	Extraction Time			Extraction Time		
	B	P			3 Hrs	6 Hrs	12 Hrs	3 Hrs	6 Hrs	12 Hrs
–	–	–	–	–	0	0	0	0	0	0
–	–	–	+	–	0	0	0	0	0	0
–	–	–	+	+	0	0	0	0	8	0
+	–	–	–	–	0	0	0	0.03	0	0
+	–	–	+	–	0	0.13	0	0	0	0
+	–	–	+	+	0	0	0	0.02	0	0.02
–	–	+	–	–	0	8	0	0.65	1.80	0.45
–	–	+	+	–	0	0	0	1.11	0	0
–	–	+	+	+	0	0	0	0.55	0.02	0.54
+	–	+	–	–	1.04	0	8.04	0.71	0.23	0
+	–	+	+	–	0.72	0.39	0.20	0.70	0.03	0
+	–	+	+	+	0.38	1.90	0.23	0.92	0.26	0.03
–	+	–	–	–	0.97	0	0.05	0.02	0.02	8
–	+	–	+	–	0.26	0.19	0.30	0.54	0	0.75
–	+	–	+	+	0.05	0	0.07	0.70	0.12	0.66
+	+	–	–	–	1.09	0.35	0.07	0.83	0	0.10
+	+	–	+	–	0.54	0.33	1.14	0.77	0.40	0
+	+	–	+	+	8.99	0.57	0.66	2.14	0.65	0

a: where, B: *B. subtilis*, P: *P. fluorescens*, Sf: Stain fungi. Buf.: 50 mM sodium acetate buffer at pH 5, TW80: Tween 80.

9.CONCLUSIONS

9.1. Total protein contents

Protein recovery from wood samples colonized by bioprotectants and/or sapstain fungi was affected by wood sample incubation time, extraction time, extraction media and pH. Under the conditions of this study, the greatest protein recovery occurred when wood samples were incubated for thirty days and extracted for twelve hours using 50 mM sodium acetate buffer at pH 5.0 with 0.05 % Tween 80. Tween 80 significantly enhanced protein recovery.

9.2. Enzyme activity

Extraction of enzymes from ponderosa pine sapwood samples colonized by bacterial bioprotectants and/or stain fungi were affected by many factors including extraction time, sample incubation period and extraction media. Under the conditions of this study, the greatest cellobiase and xylosidase activities were obtained when samples were incubated for thirty days and extracted for three hours. Increasing extraction time did not produce greater cellobiase activity. Glucosidase activity appeared to be greatest in samples exposed to microbial attack for ten days and extracted for

three hours. Longer extraction times were sometimes associated with decreased activity, suggesting that proteases were present in the extracts.

Of the three extraction media tested in this study, none of them significantly improved the activities of the enzymes evaluated. However, in some instances, activity in samples extracted in 50 mM sodium acetate buffer at pH 5.0 with 0.05% Tween 80 was slightly better than the other two extraction media.

The two bacteria, *B. subtilis* and *P. florescens* did not completely inhibit the activities of the tested sapstain fungi under the conditions of this study.

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APPENDIX

Table 1 : Analysis of variance and multiple range analysis at $\alpha = 0.05$ for protein content in extracts from ponderosa pine sapwood wafers exposed to selected bioprotectants and/or sapstain fungi for ten days and extracted for three, six or twelve hours.

Analysis of variance

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Between groups	.6862	2	.343089	.017	.9835
Within groups	1049.5884	51	20.580164		
Total (corrected)	1050.2745	53			

0 missing value(s) have been excluded.

Table of means for PROTEN.Protein by PROTEN.group

Level	Count	Average	Stnd. Error (internal)	Stnd. Error (pooled s)	95 Percent Confidence intervals for mean	
1	18	3.8522222	1.0759466	1.0692719	1.7050791	5.9993653
2	18	4.1200000	1.1401081	1.0692719	1.9728569	6.2671431
3	18	4.0444444	.9861642	1.0692719	1.8973014	6.1915875
Total	54	4.0055556	.6173444	.6173444	2.7659019	5.2452092

Multiple range analysis for PROTEN.Protein by PROTEN.group

Method: 95 Percent LSD

Level	Count	Average	Homogeneous Groups
1	18	3.8522222	X
3	18	4.0444444	X
2	18	4.1200000	X

contrast	difference	+/-	limits
1 - 2	-0.26778		3.03652
1 - 3	-0.19222		3.03652
2 - 3	0.07556		3.03652

* denotes a statistically significant difference.

Level 1: extracted for three hours.
Level 2: extracted for six hours.
Level 3: extracted for twelve hours.

Table 2: Analysis of variance and multiple range analysis at $\alpha = 0.05$ for protein content in extracts from ponderosa pine sapwood wafers exposed to selected bioprotectants and/or sapstain fungi for thirty days and extracted for three, six or twelve hours.

Analysis of variance

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Between groups	59.19787	2	29.598935	3.501	.0376
Within groups	431.14579	51	8.453839		
Total (corrected)	490.34366	53			

0 missing value(s) have been excluded.

Table of means for PROTEINB.proteinc by PROTEINB.group

Level	Count	Average	Stnd. Error (internal)	Stnd. Error (pooled s)	95 Percent Confidence intervals for mean	
1	18	2.6950000	.5265644	.6853158	1.3188570	4.0711430
2	18	3.5394444	.5609676	.6853158	2.1633015	4.9155874
3	18	5.2144444	.9038907	.6853158	3.8383015	6.5905874
Total	54	3.8162963	.3956673	.3956673	3.0217798	4.6108128

Multiple range analysis for PROTEINB.proteinc by PROTEINB.group

Method: 95 Percent LSD

Level	Count	Average	Homogeneous Groups
1	18	2.6950000	X
2	18	3.5394444	XX
3	18	5.2144444	X

contrast	difference	+/- limits	
1 - 2	-0.84444	1.94616	
1 - 3	-2.51944	1.94616	*
2 - 3	-1.67500	1.94616	

* denotes a statistically significant difference.

Level 1: extracted for three hours.
Level 2: extracted for six hours.
Level 3: extracted for twelve hours.

Table 3: Analysis of variance and multiple range analysis at $\alpha = 0.05$ for protein content in extracts from ponderosa pine sapwood wafers exposed to selected bioprotectants and/or sapstain fungi for ten days and extracted in different media.

Analysis of variance

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Between groups	466.10484	2	233.05242	20.344	.0000
Within groups	584.22844	51	11.45546		
Total (corrected)	1050.3333	53			

0 missing value(s) have been excluded.

Table of means for PROTEIND.media by PROTEINB.group

Level	Count	Average	Stnd. Error (internal)	Stnd. Error (pooled s)	95 Percent Confidence intervals for mean	
1	18	2.3250000	.8485972	.7977559	.7230726	3.9269274
2	18	1.5561111	.4397527	.7977559	-.0458163	3.1580385
3	18	8.1372222	.9978696	.7977559	6.5352948	9.7391496
Total	54	4.0061111	.4605846	.4605846	3.0812379	4.9309843

Multiple range analysis for PROTEIND.media by PROTEINB.group

Method: 95 Percent LSD

Level	Count	Average	Homogeneous Groups
2	18	1.5561111	X
1	18	2.3250000	X
3	18	8.1372222	X

contrast	difference	+/-	limits	
1 - 2	0.76889		2.26547	
1 - 3	-5.81222		2.26547	*
2 - 3	-6.58111		2.26547	*

* denotes a statistically significant difference.

Level 1: extracted in sterile distilled water(pH= 7.0).
Level 2: extracted in 50 mM sodium acetate buffer(pH= 5.0).
Level 3: extracted in 50 mM sodium acetate buffer(pH=5.0) with 0.05 % Tween 80.

Table 4: Analysis of variance and multiple range analysis at α= 0.05 for protein content in extracts from ponderosa pine sapwood wafers exposed to selected bioprotectants and/or sapstain fungi for thirty days and extracted in different extraction media.

Analysis of variance

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Between groups	113.13201	2	56.566006	7.631	.0013
Within groups	378.02892	51	7.412332		
Total (corrected)	491.16093	53			

0 missing value(s) have been excluded.

Table of means for PROTEIND.media3 by PROTEINB.group

Level	Count	Average	Stnd. Error (internal)	Stnd. Error (pooled s)	95 Percent Confidence intervals for mean	
1	18	3.1011111	.7960544	.6417135	1.8125234	4.3896989
2	18	2.5216667	.4628056	.6417135	1.2330789	3.8102544
3	18	5.8405556	.6224926	.6417135	4.5519678	7.1291433
Total	54	3.8211111	.3704935	.3704935	3.0771446	4.5650776

Multiple range analysis for PROTEIND.media3 by PROTEINB.group

Method: 95 Percent LSD

Level	Count	Average	Homogeneous Groups
2	18	2.5216667	X
1	18	3.1011111	X
3	18	5.8405556	X

contrast	difference	+/-	limits	
1 - 2	0.57944		1.82234	
1 - 3	-2.73944		1.82234	*
2 - 3	-3.31889		1.82234	*

* denotes a statistically significant difference.

Level 1: extracted in sterile distilled water(pH= 7.0).
Level 2: extracted in 50 mM sodium acetate buffer(pH= 5.0).
Level 3: extracted in 50 mM sodium acetate buffer(pH= 5.0) with 0.05 % Tween 80.

Table 5: Analysis of Variance and multiple range analysis at $\alpha = 0.05$ for Cellobiase activity in extracts from ponderosa pine sapwood wafers exposed to selected bioprotectants and/or sapstain fungi for ten days and extracted for three, six or twelve hours.

Analysis of variance

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Between groups	2.499344	2	1.2496722	1.036	.3624
Within groups	61.546989	51	1.2068037		
Total (corrected)	64.046333	53			

0 missing value(s) have been excluded.

Table of means for CELL.tendays by CELLBUFF.Group

Level	Count	Average	Stnd. Error (internal)	Stnd. Error (pooled s)	95 Percent Confidence intervals for mean	
1	18	.9311111	.3468456	.2589298	.4111691	1.4510531
2	18	.5250000	.2145207	.2589298	.0050580	1.0449420
3	18	.4372222	.1865823	.2589298	-.0827198	.9571642
Total	54	.6311111	.1494932	.1494932	.3309225	.9312998

Multiple range analysis for CELL.tendays by CELLBUFF.Group

Method: 95 Percent LSD

Level	Count	Average	Homogeneous Groups
3	18	.4372222	X
2	18	.5250000	X
1	18	.9311111	X

contrast	difference	+/-	limits
1 - 2	0.40611		0.73531
1 - 3	0.49389		0.73531
2 - 3	0.08778		0.73531

* denotes a statistically significant difference.

Level 1: extracted for three hours.
Level 2: extracted for six hours.
Level 3: extracted for twelve hours.

Table 6: Analysis of variance and multiple range analysis at $\alpha = 0.05$ for Cellobiase activity in extracts from ponderosa pine sapwood wafers exposed to selected bioprotectants and/or sapstain fungi for thirty days and extracted for three, six or twelve hours.

Analysis of variance

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Between groups	67.44740	2	33.723702	9.614	.0003
Within groups	178.89952	51	3.507834		
Total (corrected)	246.34693	53			

0 missing value(s) have been excluded.

Table of means for CELL.thirtydays by CELLBUFF.Group

Level	Count	Average	Stnd. Error (internal)	Stnd. Error (pooled s)	95 Percent Confidence intervals for mean	
1	18	2.7638889	.5714470	.4414518	1.8774352	3.6503426
2	18	1.5455556	.5074228	.4414518	.6591018	2.4320093
3	18	.0316667	.0246843	.4414518	-.8547871	.9181204
Total	54	1.4470370	.2548723	.2548723	.9352427	1.9588313

Multiple range analysis for CELL.thirtydays by CELLBUFF.Group

Method: 95 Percent LSD

Level	Count	Average	Homogeneous Groups
3	18	.0316667	X
2	18	1.5455556	X
1	18	2.7638889	X

contrast	difference	+/-	limits	
1 - 2	1.21833		1.25363	
1 - 3	2.73222		1.25363	*
2 - 3	1.51389		1.25363	*

* denotes a statistically significant difference.

Level 1: extracted for three hours.
Level 2: extracted for six hours.
Level 3: extracted for twelve hours.

Table 7: Analysis of variance and multiple range analysis at α= 0.05 for Cellobiase activity in extracts from ponderosa pine sapwood wafers exposed to selected bioprotectants and/or sapstain fungi for ten days and extracted in different extraction media.

Analysis of variance

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Between groups	1.551026	2	.7755130	.706	.4984
Within groups	56.018500	51	1.0984020		
Total (corrected)	57.569526	53			

0 missing value(s) have been excluded.

Table of means for CELLBUFF.Bufften by CELLBUFF.Group

Level	Count	Average	Stnd. Error (internal)	Stnd. Error (pooled s)	95 Percent Confidence intervals for mean	
1	18	.4094444	.2447320	.2470270	-.0865962	.9054850
2	18	.4894444	.1929500	.2470270	-.0065962	.9854850
3	18	.8022222	.2931612	.2470270	.3061816	1.2982628
Total	54	.5670370	.1426211	.1426211	.2806479	.8534262

Multiple range analysis for CELLBUFF.Bufften by CELLBUFF.Group

Method: 95 Percent LSD

Level	Count	Average	Homogeneous Groups
1	18	.4094444	X
2	18	.4894444	X
3	18	.8022222	X

contrast	difference	+/-	limits
1 - 2	-0.08000		0.70151
1 - 3	-0.39278		0.70151
2 - 3	-0.31278		0.70151

* denotes a statistically significant difference.

Level 1: extracted in sterile distilled water(pH= 7.0).
Level 2: extracted in 50 mM sodium acetate buffer(pH= 5.0).
Level 3: extracted in 50 mM sodium acetate buffer(pH= 5.0) with 0.05 % Tween 80.

Table 8: Analysis of variance and multiple range analysis at α= 0.05 for Cellobiase activity in extracts from ponderosa pine sapwood wafers exposed to selected bioprotectants and/or sapstain fungi for thirty days and extracted in different extraction media.

Analysis of variance

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Between groups	.74064	2	.3703185	.077	.9259
Within groups	244.77067	51	4.7994248		
Total (corrected)	245.51130	53			

0 missing value(s) have been excluded.

Table of means for CELLBUFF.Buffthirty by CELLBUFF.Group

Level	Count	Average	Stnd. Error (internal)	Stnd. Error (pooled s)	95 Percent Confidence intervals for mean	
1	18	1.5888889	.6324694	.5163668	.5520025	2.6257753
2	18	1.3044444	.4432237	.5163668	.2675581	2.3413308
3	18	1.4144444	.4510425	.5163668	.3775581	2.4513308
Total	54	1.4359259	.2981245	.2981245	.8372793	2.0345726

Multiple range analysis for CELLBUFF.Buffthirty by CELLBUFF.Group

Method: 95 Percent LSD

Level	Count	Average	Homogeneous Groups
2	18	1.3044444	X
3	18	1.4144444	X
1	18	1.5888889	X

contrast	difference	+/-	limits
1 - 2	0.28444		1.46638
1 - 3	0.17444		1.46638
2 - 3	-0.11000		1.46638

* denotes a statistically significant difference.

Level 1: extracted in sterile distilled water(pH= 7.0).
Level 2: extracted in 50 mM sodium acetate buffer(pH= 5.0).
Level 3: extarcted in 50 mM sodium acetate buffer(ph= 5.0) with 0.05 % Tween 80.

Table 9: Analysis of variance and multiple range analysis at $\alpha = 0.05$ for Glucosidase activity in extracts from ponderosa pine sapwood wafers exposed to selected bioprotectants and/or sapstain fungi for ten days and extracted for three, six or twelve hours.

Analysis of variance

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Between groups	1.38970	2	.6948500	.295	.7459
Within groups	120.20543	51	2.3569693		
Total (corrected)	121.59513	53			

0 missing value(s) have been excluded.

Table of means for GLU.Tendays by GLU.Group

Level	Count	Average	Stnd. Error (internal)	Stnd. Error (pooled s)	95 Percent Confidence intervals for mean	
1	18	1.2638889	.4261057	.3618601	.5372585	1.9905192
2	18	.8722222	.3342522	.3618601	.1455919	1.598852€
3	18	1.0405556	.3154957	.3618601	.3139252	1.7671859
Total	54	1.0588889	.2089200	.2089200	.6393687	1.4784091

Multiple range analysis for GLU.Tendays by GLU.Group

Method: 95 Percent LSD

Level	Count	Average	Homogeneous Groups
2	18	.8722222	X
3	18	1.0405556	X
1	18	1.2638889	X

contrast	difference	+/-	limits
1 - 2	0.39167		1.02761
1 - 3	0.22333		1.02761
2 - 3	-0.16833		1.02761

* denotes a statistically significant difference.

Level 1: extracted for three hours.
Level 2: extracted for six hours.
Level 3: extracted for twelve hours:

Table 10: Analysis of variance and multiple range analysis at $\alpha = 0.05$ for Glucosidase activity in extracts from ponderosa pine sapwood wafers exposed to selected bioprotectants and/or sapstain fungi for thirty days and extracted for three, six or twelve hours.

Analysis of variance

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Between groups	1.042831	2	.5214156	1.292	.2836
Within groups	20.584698	51	.4036215		
Total (corrected)	21.627529	53			

0 missing value(s) have been excluded.

Table of means for GLU.Thirtydays by GLU.Group

Level	Count	Average	Stnd. Error (internal)	Stnd. Error (pooled s)	95 Percent Confidence intervals for mean	
1	18	.6194444	.1412606	.1497445	.3187512	.9201377
2	18	.2850000	.1051244	.1497445	-.0156933	.5856933
3	18	.5071111	.1904325	.1497445	.2064178	.8078044
Total	54	.4705185	.0864550	.0864550	.2969132	.6441239

Multiple range analysis for GLU.Thirtydays by GLU.Group

Method: 95 Percent LSD

Level	Count	Average	Homogeneous Groups
2	18	.2850000	X
3	18	.5071111	X
1	18	.6194444	X

contrast	difference	+/-	limits
1 - 2	0.33444		0.42524
1 - 3	0.11233		0.42524
2 - 3	-0.22211		0.42524

* denotes a statistically significant difference.

Level 1: extracted for three hours.
Level 2: extracted for six hours.
Level 3: extracted for twelve hours.

Table 11: analysis of variance and multiple range analysis at α= 0.05 for Glucosidase activity in extracts from ponderosa pine sapwood wafers exposed to selected bioprotectants and/or sapstain fungi for ten days and extracted in different extraction media.

Analysis of variance

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Between groups	.424226	2	.2121130	.114	.8925
Within groups	94.896828	51	1.8607221		
Total (corrected)	95.321054	53			

0 missing value(s) have been excluded.

Table of means for GLUBUFF.Bufften by GLUBUFF.Group

Level	Count	Average	Stnd. Error (internal)	Stnd. Error (pooled s)	95 Percent Confidence intervals for mean	
1	18	.8488889	.3326747	.3215174	.2032684	1.4945094
2	18	.9983333	.2910374	.3215174	.3527128	1.6439539
3	18	1.0600000	.3387405	.3215174	.4143795	1.7056205
Total	54	.9690741	.1856282	.1856282	.5963249	1.3418233

Multiple range analysis for GLUBUFF.Bufften by GLUBUFF.Group

Method: 95 Percent LSD

Level	Count	Average	Homogeneous Groups
1	18	.8488889	X
2	18	.9983333	X
3	18	1.0600000	X

contrast	difference	+/-	limits
1 - 2	-0.14944		0.91305
1 - 3	-0.21111		0.91305
2 - 3	-0.06167		0.91305

* denotes a statistically significant difference.

Level 1: extracted in sterile distilled water(pH= 7.0).
Level 2: extracted in 50 mM sodium acetate buffer(pH= 5.0).
Level 3: extracted in 50 mM sodium acetate buffer(pH= 5.0) with 0.05 % Tween 80.

Table 12: Analysis of variance and multiple range analysis at α= 0.05 for Glucosidase activity in extracts from ponderosa pine sapwood wafers exposed to selected bioprotectants and/or sapstain fungi for thirty days and extracted in different extraction media.

Analysis of variance

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Between groups	.185200	2	.0926000	.220	.8033
Within groups	21.472683	51	.4210330		
Total (corrected)	21.657883	53			

0 missing value(s) have been excluded.

Table of means for GLUBUFF.Buffthirty by GLUBUFF.Group

Level	Count	Average	Stnd. Error (internal)	Stnd. Error (pooled s)	95 Percent Confidence intervals for mean	
1	18	.4727778	.1726756	.1529403	.1656673	.7798883
2	18	.4061111	.1241734	.1529403	.0990006	.7132216
3	18	.5494444	.1579122	.1529403	.2423340	.8565549
Total	54	.4761111	.0883001	.0883001	.2988008	.6534214

Multiple range analysis for GLUBUFF.Buffthirty by GLUBUFF.Group

Method: 95 Percent LSD

Level	Count	Average	Homogeneous Groups
2	18	.4061111	X
1	18	.4727778	X
3	18	.5494444	X

contrast	difference	+/-	limits
1 - 2	0.06667		0.43432
1 - 3	-0.07667		0.43432
2 - 3	-0.14333		0.43432

* denotes a statistically significant difference.

Level 1: extracted in sterile distilled water(pH= 7.0).
Level 2: extracted in 50 mM sodium acetate buffer(pH= 5.0).
Level 3: extracted in 50 mM sodium acetate buffer(pH= 5.0) with 0.05 % Tween 80.

Table 13: Analysis of variance and multiple range analysis at $\alpha = 0.05$ for xylosidase activity in extracts from ponderosa pins sapwood wafers exposed to selected bioprotectants and/or sapstain fungi for ten days and extracted for three, six or twelve hours.

Analysis of variance

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Between groups	.3096444	2	.1548222	.958	.3904
Within groups	8.2408889	51	.1615861		
Total (corrected)	8.5505333	53			

0 missing value(s) have been excluded.

Table of means for XY.Tendays by XY.Group

Level	Count	Average	Stnd. Error (internal)	Stnd. Error (pooled s)	95 Percent Confidence intervals for mean	
1	18	.3355556	.1020772	.0947470	.1452995	.5258116
2	18	.2144444	.1076129	.0947470	.0241884	.4047005
3	18	.1533333	.0702191	.0947470	-.0369227	.3435894
Total	54	.2344444	.0547022	.0547022	.1246001	.3442888

Multiple range analysis for XY.Tendays by XY.Group

Method: 95 Percent LSD

Level	Count	Average	Homogeneous Groups
3	18	.1533333	X
2	18	.2144444	X
1	18	.3355556	X

contrast	difference	+/-	limits
1 - 2	0.12111		0.26906
1 - 3	0.18222		0.26906
2 - 3	0.06111		0.26906

* denotes a statistically significant difference.

Level 1: extracted for three hours.
Level 2: extracted for six hours.
Level 3: extracted for twelve hours.

Table 14: Analysis of variance and multiple range analysis at α= 0.05 for Xylosidase activity in extracts from ponderosa pine sapwood wafers exposed to selected bioprotectants and/or sapstain fungi for thirty days and extracted for three, six or twelve hours.

Analysis of variance

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Between groups	1.6645481	2	.8322741	4.405	.0172
Within groups	9.6349278	51	.1889202		
Total (corrected)	11.299476	53			

0 missing value(s) have been excluded.

Table of means for XY.Thirtydays by XY.Group

Level	Count	Average	Stnd. Error (internal)	Stnd. Error (pooled s)	95 Percent Confidence intervals for mean	
1	18	.5383333	.1306826	.1024479	.3326137	.7440530
2	18	.1961111	.1033243	.1024479	-.0096085	.4018307
3	18	.1416667	.0610970	.1024479	-.0640530	.3473863
Total	54	.2920370	.0591483	.0591483	.1732648	.4108093

Multiple range analysis for XY.Thirtydays by XY.Group

Method: 95 Percent LSD

Level	Count	Average	Homogeneous Groups
3	18	.1416667	X
2	18	.1961111	X
1	18	.5383333	X

contrast	difference	+/-	limits	
1 - 2	0.34222		0.29093	*
1 - 3	0.39667		0.29093	*
2 - 3	0.05444		0.29093	

* denotes a statistically significant difference.

Level 1: extracted for three hours.
Level 2: extracted for six hours.
Level 3: extracted for twelve hours.

Table 15: Analysis of variance and multiple range analysis at α= 0.05 for Xylosidase activity in extracts from ponderosa pine sapwood wafers exposed to selected bioprotectants and/or sapstain fungi for then days and extracted in different extraction media.

Analysis of variance

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Between groups	.0427444	2	.0213722	.128	.8800
Within groups	8.5077889	51	.1668194		
Total (corrected)	8.5505333	53			

0 missing value(s) have been excluded.

Table of means for XYBUFF.Bufften by XYBUFF.Group

Level	Count	Average	Stnd. Error (internal)	Stnd. Error (pooled s)	95 Percent Confidence intervals for mean	
1	18	.2005556	.0924979	.0962691	.0072431	.3938680
2	18	.2333333	.0732352	.0962691	.0400209	.4266458
3	18	.2694444	.1178303	.0962691	.0761320	.4627569
Total	54	.2344444	.0555810	.0555810	.1228354	.3460534

Multiple range analysis for XYBUFF.Bufften by XYBUFF.Group

Method: 95 Percent LSD

Level	Count	Average	Homogeneous Groups
1	18	.2005556	X
2	18	.2333333	X
3	18	.2694444	X

contrast	difference	+/-	limits
1 - 2	-0.03278		0.27339
1 - 3	-0.06889		0.27339
2 - 3	-0.03611		0.27339

* denotes a statistically significant difference.

Level 1: extracted in sterile distilled water(pH= 7.0).
Level 2: extracted in 50 mM sodium acetate buffer(pH= 5.0).
Level 3: extracted in 50 mM sodium acetate buffer(pH= 5.0) with 0.05 % Tween 80.

Table 16: Analysis of variance and multiple range analysis at α= 0.05 for Xylosidase activity in extracts from ponderosa pine sapwood wafers exposed to selected bioprotectants and/or sapstain fungi for thirty days and extracted in different extraction media.

Analysis of variance

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Between groups	.087659	2	.0438296	.199	.8200
Within groups	11.220400	51	.2200078		
Total (corrected)	11.308059	53			

0 missing value(s) have been excluded.

Table of means for XYBUFF.Buffthirty by XYBUFF.Group

Level	Count	Average	Stnd. Error (internal)	Stnd. Error (pooled s)	95 Percent Confidence intervals for mean	
1	18	.2577778	.1125247	.1105561	.0357764	.4797791
2	18	.2388889	.0867492	.1105561	.0168875	.4608903
3	18	.3322222	.1283774	.1105561	.1102209	.5542236
Total	54	.2762963	.0638296	.0638296	.1481237	.4044688

Multiple range analysis for XYBUFF.Buffthirty by XYBUFF.Group

Method: 95 Percent LSD

Level	Count	Average	Homogeneous Groups
2	18	.2388889	X
1	18	.2577778	X
3	18	.3322222	X

contrast	difference	+/-	limits
1 - 2	0.01889		0.31396
1 - 3	-0.07444		0.31396
2 - 3	-0.09333		0.31396

* denotes a statistically significant difference.

Level 1: extracted in sterile distilled water(pH= 7.0).
Level 2: extracted in 50 mM sodium acetate buffer(pH= 5.0).
Level 3: extracted in 50 mM sodium acetate buffer(pH= 5.0) with 0.05 % Tween 80.