EFFECT OF COMPRESSION OF GREEN WOOD CHIPS ON CONIDIAL GERMINATION AND COLONIZATION OF A BIOPULPING FUNGUS, *PHANEROCHAETE CHRYSOSPORIUM*

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ABSTRACT

Compression and baling of green wood ships inoculated with a biopulping fungus *Phanerochaete chrysosporium* has produced pulps with increased strength properties and reduced energy inputs without the need for steaming of chips or specialized bioreactor conditions. Use of a contact-agar method to study spore germination has shown that compression of green wood enhances rates of sapwood colonization by two strains of this white-rot fungus. This response was verified by SEM observation and is thought to occur as a result of parenchyma death during chip compression. The colonization of this fungus on softwood chips was also improved as a result of compression.

Keywords: Compression-baling, biopulping, *Phanerochaete chrysosporium,* parenchyma cells, germination, colonization, contact-agar method, SEM, aspen, jack pine, balsam fir.

INTRODUCTION

In nature, white-rot fungi possess the ability to decompose the cell-wall structural polymers: cellulose, hemicellulose, and lignin. Several white-rot fungi that selectively degrade lignin without deteriorating cellulose have been explored in several biotechnology areas. Among them, biopulping, which is defined as inoculating pulpwood with white-rot fungi prior to conventional pulping processes, has received much attention in the past two decades. The research has focused on the use of fungal pretreatment in mechanical pulping or as an alternative to chemical pretreatments in high-yield pulping where savings in electrical energy and improvements in the properties of resultant pulp and paper, e.g., increased burst, tensile, and tear strengths, are reported although decreases in brightness and light scattering coefficient are also encountered (Akhtar 1994; Bar-Lev et al. 1982; Eriksson and Vallander 1982; Kashino et al. 1993; Kirk et al. 1993; Setliff et al. 1990). Numerous fungi have been investigated for this application. Among them, *Phanerochaete chrysosporium* Burds was found to selectively degrade lignin in the incipient stages and is capable of competing with naturally occurring microorganisms in chip piles. *P. chrysosporium* also produces abundant quantities of conidia, which makes manipulation of inoculation easier (Kirk et al. 1993). Moreover, the

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optimal growth temperature of this fungus is close to the temperature inside a chip pile. These factors make this fungus suitable for biopulping purposes. Indeed, this fungus has been extensively studied for its lignin-degrading capabilities and applications. For these reasons, *P. chrysosporium* was chosen for this study.

Compression dewatering and baling of green wood chips was first investigated for the use of wood chips as industrial energy sources (Steklenski et al. 1989). In the process, it was noted that *P. chrysosporium* had developed naturally inside the chip bales. This technique was then explored as a potential incubation method in biopulping applications. It was later reported that paper strength properties (e.g., burst) of kraft and refiner pulps were improved after fungal treatment with this incubation technique where neither heat treatment of wood nor controlled conditions were employed (Chen and Schmidt 1995; Schmidt et al. 1994).

The compression-baling technique has three characteristics that are thought to provide a favorable environment for this fungus, *P. chrysosporium,* to compete with other naturally occurring microorganisms. The direct effect of the compression is a lowered moisture content due to the mechanical forces applied on the chips which squeeze free water from the cell lumens. The moisture content of green wood was reported to decrease from approximately 102% to a 60% range (dry basis) after compression (Haygreen 1981; Steklenski et al. 1989). This moisture content range is advantageous for fungal growth. However, moisture content reduction is unlikely to be the sole factor responsible for the favorable conditions promoting bale colonization by *P. chrysosporium.* Another important effect is the rise of temperature in bales. The temperature inside an insulated compressed bale could reach 40°C within 24 h after baling and stay higher than ambient temperature throughout storage (Lin and Schmidt 1991; Steklenski et al. 1989). This initial temperature rise is believed to result mainly from the respiration of dying parenchyma cells and the metabolic activities of bacteria (Springer and Hajny 1970). This internal temperature rise observed in compressed bales is favorable for the growth of thermotolerant microorganisms such as *P. chrysosporium,* while it may be detrimental for most microorganisms commonly found in chip piles.

Another important effect of compression of green chips is the decrease in the number of viable parenchyma cells. Using TTC (1% triphenyltetrazolium chloride solution) to determine the viability of parenchyma cells, Lin and Schmidt (1991) reported a substantial reduction in the stained areas on wood chips after compression, indicating a significant loss of viable parenchyma cells. It is known that food reserves (e.g., starch) in parenchyma cells are converted to antimicrobial compounds such as tannins in response to injury (Wardell and Hart 1970). Shortle and Cowling (1978) reported that bacteria and nonhymenomycetous fungi were able to grow on live sapwood, whereas the growth of decay fungi on live sapwood was limited due to intolerance toward antimicrobial compounds. However, decay fungi eventually out-competed other microorganisms when the readily metabolizable nutrients became depleted. All tested decay fungi grew better on rapidly killed sapwood (no viable parenchyma cells) than on live sapwood. This concurs with the report by Lin and Schmidt (1991), who observed that in the early stage of storage, abundant bacteria along with some basidiomycetes colonized compressed chips (less viable parenchyma cells) while phycomycetes and *Fungi imperfecti* dominated noncompressed chips. Reid (1989) noted a lag period of 2–6 days during solid-state fermentation of aspen (*Populus tremuloides* Michx.) with *Phlebia tremellosa* (Schrad.:Fr.) Nakas, et Burds. and proposed that either certain inhibitors present in the wood must be destroyed or fungal metabolites must accumulate before rapid growth can occur. As far as the first of the two alternatives is concerned, living parenchyma cells might be responsible for the observed delay. This

supports the idea that the decrease in viable parenchyma cells improves colonization by certain fungi, such as basidiomycetes.

Breuil and coworkers (1994) reported that nondecay fungi (early wood colonizers) grew poorly on aspen and lodgepole pine (*Pinus contorta var. latifolia*) heartwood chips. The level of ergosterol detected in the heartwood was at best only a third of the level in sapwood after two weeks of fungal growth. Studying reasons responsible for poor fungal growth on heartwood, they concluded that moisture content, nitrogen content, or fatty acids were unlikely to be the factors while low levels of triglycerides in the heartwood of both species and high amounts of steryl esters and waxes in aspen heartwood and resin acids in lodgepole pine could explain this phenomenon. It was therefore of interest to explore whether compression of chips results in redistribution of cell contents in the chip mass and a consequent improvement in the development of microorganisms on heartwood.

It has been documented that the germination of spores of decay fungi on wood is sensitive to chemical modifications of the wood. Wood species, sapwood/heartwood, sterilization methods, and prior extraction all have significant influences on spore germination (Schmidt and French 1979). Therefore, the changes caused by compression of wood chips in the bale-making process ("lower" moisture content, availability of nutrients, viability of parenchyma cells, presence of antimicrobial compounds, and the consequent temperature rise in bales) might be expected to affect conidial germination and subsequent colonization of *P. chrysosporium.*

The germination of fungal spores has been widely used as an experimental process for studying the mechanisms and environmental conditions involved in the transition from dormancy to active metabolism and growth. There are several ways of monitoring germination, such as measurements of spore diameters and particle-size distributions. Due to its simplicity, the emergence of a germ-tube has been the most commonly used criterion (Carlile and Watkinson 1994). In this study, this method was used to investigate the effects of compression of aspen green chips on the germination of *P. chrysosporium* conidia.

The purpose of this research was to determine whether compression of green aspen chips would increase the germination rate of *P. chrysosporium* conidia on agar plugs fused to sapwood or heartwood chips. This would help in understanding the reasons behind the successful colonization of nonsterile aspen chips by *P. chrysosporium* in stored bales as previously documented. Results from this contact-agar method were then confirmed by SEM examination of chip colonization. In addition, aspen-expressed liquid obtained in the process of compression was examined for possible enhancement of conidial germination *in vitro.* We also studied the effects of compression on visible fungal surface development on softwood chips (jack pine, *Pinus banksiana* Lamb., and balsam fir, *Abies balsamea* [Linnaeus] Miller).

MATERIALS AND METHODS

Fungi

The *P. chrysosporium* strains used in conidial germination study were ATCC 24725 and ATCC 28326. The former is the most extensively explored strain in biopulping and other lignin-degrading studies. This fungal strain is capable of growing on nonsterile hardwoods (Kirk et al. 1993), whereas the growth on softwoods is limited. On the contrary, the later strain was first isolated from pine chips. These two strains were therefore used in the conidial germination study to examine if differences in germination on aspen and the effects of compression of chips existed. In the second part of study, visual comparison of fungal development on softwoods, *P. chrysosporium* strains ATCC 28326 and Forintek A436 were used since ATCC 24725 did not show surface mycelial development on softwood chips in preliminary screening trials (Olsen 1996).

I. CONIDIAL GERMINATION STUDY

Agar plate method

Thirty-five-mm diameter and 3-mm-thick 2% purified water agar (BBL®, BBL Microbiology Systems, Cockeysville, MD) plates were prepared and kept at 24°C or 34°C and 75% relative humidity for 24 h before conidial suspension was added. A conidial suspension of *P. chrysosporium* strain (ATCC 24725 or ATCC 28326) was prepared from 9-day-old malt extract agar plates (1.5% malt extract and 2% agar). Conidia were recovered by gently shaking the colony with a mixture of sterile deionized water and Tween 80 (one drop of Tween 80 per liter of sterile water). The concentration of conidial suspension was assessed using white cell count method on a hemacytometer (American Optical, Buffalo, NY) and adjusted to 1×10^6 per ml (the inoculum amount used in previous aspen chip bale studies). Two hundred μl of the conidial suspension were added to each agar plate. The inoculated agar plates were kept at 75% relative humidity and a temperature of 24 or 34°C. In one study, various concentrations of aspen-expressed liquid in conidial suspensions (0, 33, 50, 67, 100%) were examined for their effects on germination and to determine the optimal concentration for further studies. After 9.5 h, a cover glass was placed onto each inoculated agar plate and the plates were then observed under light microscope (×200) for germination assessment. A conidium was considered germinated if the germ tube exceeded the diameter of the conidium. Germination rates were calculated as the percentage of germinated conidia over 200 conidia examined from randomly chosen fields on each agar plate. The germination rate for each set was obtained from the average of three observed agar plates.

Chip preparations

Sapwood and heartwood of debarked fresh aspen were separated from a band saw and chipped using an Appleton disk chipper and screening apparatus. Chips were then pressed at 34.5 MPa (5000 psi) for 5 min with a hydraulic ram press, which corresponded to the compression conditions used for bale-making in prior studies (Chen and Schmidt 1995). Noncompressed sapwood and heartwood chips were used as controls.

Contact-agar method

Primary studies on conidial germination on contact-agar discs were carried out on noncompressed and sterilized aspen sapwood and heartwood chips (chips were steam-sterilized at 100°C for 10 min in an autoclave). It was first examined whether aspen sapwood and heartwood chips resulted in differences in germination; thus no aspen-expressed liquid was added into the conidial suspension. Aspen-expressed liquid was added in later studies to investigate its influence on germination on sapwood and heartwood chips. Agar plugs (7 mm in diameter × 3 mm thick) were cut from purified agar plates and attached onto chips with one drop of molten agar. This contact-agar method was reported to better represent the germination of fungal spores on wood surface than other methods. As the liquid agar cooled, the agar plug was firmly fused to the wood surface and the chemical solution within the wood sample was able to diffuse into agar. Consequently, the influence of the chemical components on germination was taken into account (Schmidt and French 1977). The wood chip with the sterile water agar plugs fused to its surface was placed upon a sterile glass slide on the top of a moistened filter paper inside a petri dish. This assemblage was held for 24 h before adding conidial suspension to allow diffusion of water-soluble components from wood chips to agar plugs. Twenty μl of conidial suspension (prepared as that used in agar plate studies) with or without the addition of aspen-expressed liquid were deposited on each agar plug. After 9.5 h of incubation at 34°C, the agar plugs were removed to a glass slide, and observed under light microscope (×200). Germination rates were calculated as the percentage of germinated conidia from 200 conidia examined on each plug. The germi-

TABLE 1. *Effects of temperature on conidial germination on agar plates after 9.5 hours.* [1]

	Phanerochaete chrysosporium strains			
	ATCC 24725		ATCC 28326	
Temperature	34°C	24°C	34°C	24°C
Germination	36.27 (2.72)	6.08 (1.24)	19.79 (1.69)	2.00 (0.94)

[1] The values represent the average germination rates (3 replicates with total of 600 conidia observed). The values in parentheses are the standard deviations.

nation rate for each set was obtained from the average of three agar plugs from three wood chips.

Scanning electron microscopy

In order to better characterize and confirm results quantified in the contact-agar method, approximately 2 ml of conidial suspension of *P. chrysosporium* ATCC 24725 with the density of 1×10^6 per ml was directly sprayed onto wood chips until the surfaces of chips were moist. Four inoculated chips for each set were incubated at 34°C for 12 h. Two chips were randomly selected for SEM observation and fixed in 1% osmium tetroxide for 4 h. Afterwards, the chips were dehydrated in an ascending dilution series of ethanol (25%, 50%, 70%, 95%, and 100%) and then substituted with absolute acetone (Read et al. 1992). The material was critical point dried (Critical Point Dryer, LADD Research Industries), mounted onto aluminum stubs, coated with 40% gold/60% palladium in a vacuum evaporator, and observed with a Philips 500× scanning electron microscope. Both chips from each set were scanned, and one field that best represented the extent of conidial germination on the individual chip was photographed with Polaroid type 55 film at the magnification of 160.

II. VISUAL COMPARISON OF FUNGAL DEVELOPMENT ON SOFTWOODS

Fresh jack pine and balsam fir logs were debarked and chipped. The moisture contents of noncompressed chips of both species were determined immediately in triplicate. A proportion of chips were compressed at 34.5 MPa (5000 psi) for 5 min. The potential effect on moisture on fungal colonization was eliminated by adjusting compressed chips to the original noncompressed moisture content for each wood species. One hundred grams of noncompressed or compressed chips were placed in plastic storage bags and then inoculated with 30 ml of a conidial suspension (10^6 conidia per ml) of respective fungal strains (*P. chrysosporium* ATCC 28326 and Forintek A436). Four replicate bags were prepared per treatment on each wood species. The bags were stored in a controlled chamber maintained at 29°C and 75% relative humidity for two weeks.

At the end of incubation, the inoculated wood chips in the plastic bags were spread out, and the surfaces of forty randomly chosen wood chips were examined for any visible sign of mycelial development typical of *P. chrysosporium.* Isolation of the surface mycelium onto common media confirmed the fungus identity. The number of chips of the forty that had any visible growth was recorded, and the resulting number was used to compare the rates of fungal development.

RESULTS AND DISCUSSION

I. CONIDIAL GERMINATION

Agar plate method

Table 1 indicates that both strains of *P. chrysosporium* germinated at higher percentages and higher temperature, which was anticipated since this fungus is known to be thermotolerant. This confirms the suspicion that the early temperature rise in bales contributes to providing a favorable condition for *P. chrysosporium* to develop. Later studies were therefore carried out at 34°C.

The effect of aspen-expressed liquid at various concentration levels on conidial germi-

TABLE 2. *Effects of aspen-expressed liquid on conidial germination on agar plates.* [1]

	Aspen-expressed liquid concentration				
	0%	33%	50%	67%	100%
ATCC 24725	3.80 (0.35)	78.60 (1.37)	86.34 (0.89)	77.33 (0.46)	65.97 (1.82)
ATCC 28326	29.47 (0.88)	86.13 (2.15)	69.35 (3.44)	70.34 (2.53)	29.05 (1.10)

[1] The values represent the average germination rates (3 replicates with total of 600 conidia observed) after 9.5 hours. The values in parentheses are the standard deviations.

nation are presented in Table 2. The concentration range of 33 to 67% led to higher germination of both *P. chrysosporium* strains, indicating that aspen-expressed liquid did stimulate conidial germination. For simplicity, the concentration of 50% was used in later studies.

Contact-agar method

Two separate trials (sets 1 and 2) were carried out on noncompressed, sterilized aspen chips. The data indicated that the conidia of both strains germinated more rapidly on sapwood than on heartwood chips (Table 3). These observations confirmed that the germination on heartwood is limited most likely due to either the lack of nutrient or the presence of toxic compounds. There was not substantial difference in germination on sapwood between these two strains. However, it was noted that strain ATCC 28326 had a higher germination rate on heartwood chips than did ATCC 24725.

Germination testing was then carried out on nonsterilized chips because sterilization proved to be unnecessary for germination to occur as evidenced from previous bale studies using nonsterile inoculated chips. As for sterilized chips, sapwood supported higher germination rates than did heartwood in both compressed and noncompressed cases. Table 4 shows that compression profoundly enhanced the germination on sapwood chips. The strain ATCC 28326 (first isolated from a *Pinus* chip pile in Sweden) had poor germination rates on both noncompressed sapwood and heartwood chips. It might indicate that this strain was more sensitive to chemical components present in fresh aspen chips. Compression substantially improved germination of this strain on sapwood chips. Nevertheless, we expected that compression would also increase germination on heartwood, for aspen-expressed liquid from sapwood chips presumably coated chips during compression, which increased the nutrient level on heartwood chips. This improvement was not observed. It was possible that coating of the expressed liquid during compression was not sufficient to stimulate conidial germination. To examine its direct effect on conidial germination, aspen-expressed

TABLE 3. *Germination rates of* P. chrysosporium *conidia on contact-agar discs attached to noncompressed sterilized aspen chips.* [1]

	Phanerochaete chrysosporium strains	
	ATCC 24725	ATCC 28326
Set 1		
Sapwood	88.27 (2.85)	86.14 (1.13)
Heartwood	13.48 (1.76)	49.50 (3.87)
Set 2		
Sapwood	81.00 (1.65)	77.20 (3.42)
Heartwood	21.23 (1.87)	45.68 (2.44)

[1] The values represent the average germination rates (3 replicates with total of 600 conidia observed) after 9.5 hours of incubation at 34°C. The values in parentheses are the standard deviations.

TABLE 4. *Effects of compression on germination rates on nonsterilized chips.* [1,2,3]

	Phanerochaete chrysosporium strains	
	ATCC 24725	ATCC 28326
Sapwood-C	63.30 (6.23)[a]	81.37 (6.15)[a]
Sapwood-NC	26.45 (3.42)[b]	5.20 (1.31)[b]
Heartwood-C	10.21 (5.68)[b]	2.79 (0.63)[b]
Heartwood-NC	18.88 (5.24)[b]	0.33 (0.24)[b]

[1] The values represent the average germination rates (3 replicates with total of 600 conidia observed). The values in parentheses are the standard deviations.

[2] C = compressed; NC = noncompressed.

[3] The same letters within the column indicate the homogeneous group determined by Least Significant Difference pairwise comparisons ($\alpha = 0.05$).

TABLE 5. *Effect of the addition of aspen-expressed liquid on germination rates on compressed, nonsterilized chips.* [1,2,3]

	Phanerochaete chrysosporium strains	
	ATCC 24725	ATCC 28326
Sapwood-control	79.32 (6.87)[ab]	97.83 (0.85)[a]
Sapwood-AEL	91.87 (1.39)[ab]	98.84 (0.24)[a]
Heartwood-control	58.17 (8.06)[c]	2.82 (0.48)[c]
Heartwood-AEL	76.47 (4.92)[b]	19.59 (2.10)[b]

[1] The values represent the average germination rates (3 replicates with total of 600 conidia observed). The values in parentheses are the standard deviations.
[2] control = No aspen-expressed liquid addition; AEL = addition of aspen-expressed liquid into conidial suspension.
[3] The same letters within the column indicate the homogeneous group determined by Least Significant Difference pairwise comparisons (α = 0.05).

liquid was then added into conidial suspensions (50% concentration based on previous studies on agar plates shown in Table 2). Table 5 indicates that aspen-expressed liquid did not significantly improve the germination rates on compressed sapwood chips as the germination rates of both strains were already high without this addition. However, the germination on compressed heartwood chips was improved by the presence of aspen-expressed liquid. This enhancement was more obvious for strain ATCC 24725, a strain known to colonize aspen effectively, but aspen-expressed liquid proved to be beneficial to conidial germination of both fungal strains.

The germination study was also carried out at 27°C which is more optimal for other commonly found antagonistic microorganisms such as *Trichoderma spp.* The results (data not shown) indicated that the effects of compression were similar to those at 34°C. Within the first 12 h after compression/baling, the temperature at some areas inside the bale could still be in this mesophilic range. Consequently, the data presented here could characterize the onset of conidial germination in chip bales.

FIG. 2. The surface of noncompressed aspen sapwood chip 12 hours after seeding conidia shows few developing hyphae (×160).

FIG. 1. Germinated conidia on the surface of compressed aspen sapwood chip after 12 hours of incubation (×160).

Scanning electron microscopy

The increased conidial germination of ATCC 24725 on compressed aspen sapwood noted in the contact-agar trials was confirmed by SEM observation of chips after 12 h of incubation (Figs. 1 and 2). The hyphae on compressed sapwood (Fig. 1) reached 100 μ, whereas only few developing hyphae were noted on noncompressed chips (Fig. 2). Similar to the results from contact-agar method, the germination on compressed heartwood chips was much more limited than sapwood, although few germinated conidia with short hyphae were noted. On the other hand, noncompressed heartwood showed no sign of conidial germination.

TABLE 6. *Visual comparison of fungal development on softwood chips as expressed by chip count data at 2 weeks' growth.* [1,2]

	Phanerochaete chrysosporium strains			
	ATCC 28326		Forintek A436	
	Non-compression	Compression	Non-compression	Compression
Jack pine	0.0 (0.0)[a]	37 (8.8)[b]	1.9 (2.4)[a]	29 (6.6)[b]
Balsam fir	0.0 (0.0)[a]	18 (5.4)[b]	3.1 (3.1)[a]	14 (3.1)[b]

[1] Means are the percent fraction of the 40 chips counted in each bag (n = 4) that exhibited observable mycelial growth. The values in parentheses are the standard deviations.
[2] The same letters within the row indicate the homogeneous group determined by Bonferroni multiple comparisons procedure ($\alpha = 0.05$)

II. VISUAL COMPARISON OF FUNGAL DEVELOPMENT ON SOFTWOODS

A significant increase in growth was noted for each fungal strain as a result of compression (Table 6). Strain ATCC 28326 did not show any sign of growth on noncompressed chips from both wood species, whereas significant mycelial development was noted on compressed chips. The compressed chips had visibly colonized chip counts four and fifteen times higher than noncompressed for Forintek A436 strain on balsam fir and jack pine, respectively. At the end of two weeks, ATCC 28326 (strain from pine) more completely colonized both wood species than did Forintek A436 (hardwood isolate). After compression, jack pine supported growth of both *P. chrysosporium* strains more readily than balsam fir. The results confirmed our postulation that the changes in softwood chips due to compression would promote the early colonization of inoculated fungus. For these two softwood species, this was true even when moisture contents of compressed and noncompressed chips were the same.

CONCLUSIONS

The results of this study showed that the modifications of aspen chips after compression led to more conidial germination of examined *P. chrysosporium* strains on sapwood. Although the beneficial effects of compression on heartwood chips was limited for conidial germination, the addition of aspen-expressed liquid that was obtained in the process of compression into conidial suspension did improve germination observed by means of agar plate and contact-agar methods. Mycelial development of two *P. chrysosporium* strains on softwood chips was also improved by compression, which merits attention since *P. chrysosporium* is generally known to be an ineffective softwood colonizer. The enhancement seen in this study could be the combined effects of three mechanisms described above as well as the presence of extractives. It can be concluded that this compression/baling technique provides an environment that is favorable for the early establishment of *P. chrysosporium* on both hardwood and softwood chips. The death of parenchyma cells by compression would seem to provide the most influential improvement to early chip colonization by *P. chrysosporium* strains.

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