EXTRACELLULAR PROTEINS PRODUCED BY DIFFERENT SPECIES OF THE FUNGUS *TRICHODERMA* ON A SECONDARY PAPER MILL SLUDGE SUBSTRATE

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Kraft pulping is the most commonly used pulping process in the pulp and paper industry. In this process wood chips are chemically delignified using sodium sulfide and sodium hydroxide. Delignification is usually followed by mechanical fiberization and a bleaching process of the resulting wood pulp. In addition to lignin-free wood pulp, this process also produces waste that contains residues of used chemicals, lignin, cellulose, hemicelluloses, and small amounts of other wood components. Because of the worldwide large-scale production of paper, the sludge from paper mills contributes significantly to environmental pollution. Although there have been great efforts being made to utilize this lignin-rich material, sludge is mostly disposed in landfills or incinerated in a boiler. This research project used secondary sludge as a substrate for 7 wood-decay fungi taxonomically belonging to the genus *Trichoderma*. The examined fungi expressed the capability of consuming sludge components as a carbon source to produce extracellular proteins. The proteins were separated by gel electrophoresis. Before and after fungi cultivation, the sludge was analyzed by Fourier transform infrared spectroscopy (FTIR).

Keywords: Secondary paper mill sludge; Wood decay fungi; Trichoderma; Extracellular proteins

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INTRODUCTION

The current large consumption and production of paper results in enormous amounts of paper mill waste. Global production of paper mill sludge is predicted to rise over the next 50 years by between 48 and 86%. This could have large ramifications in terms of landfill space and limited incinerator capacities (Mabee and Roy 2003). Considering this prognosis, in the future, paper mill sludge will either be an alarming environmental problem or an abundant raw material for new alternative material production. Future uses of sludge will depend on results of studies that investigate its possible beneficial use. Currently, primary and secondary paper mill sludge is merely disposed of in landfills without beneficial use.

Sludge is a complex mixture of chemicals and substances that are potentially useful in industry applications; for instance, sludge contains valuable carbohydrates. The process of extracting desired substances from sludge is not effective due to the technological and economical requirements of such a demanding process. Another alternative to taking advantage of this abundant, available, and cheap substance is using

the sludge for the biological generation of valuable organic compounds, such as proteins and lipids. This approach of treating the waste can also intensify the utilization of the primary raw material resources, in regards to the protection of the natural environment.

There are two main techniques of treating and utilizing paper mill sludge. The first technique is using the fibers in the sludge as the mechanical reinforcement in composite materials (Lee at al. 1999; Fernandez et al. 2001; Taramian et al. 2007); however, the removal of impurities and excess water are not economical. The second technique includes modifying the sludge chemically by various biological organisms, followed by chemical treatment. In addition, incineration of the solid sludge for energy production is widely used, but this comes with a costly drying process and a threat of air pollution. A small part of paper mill waste sludge is used in land-spreading applications for soil enrichment. Paper mills that own woodlands have discovered that land spreading and composting of dried sludge can yield benefits in terms of the nutritional value of the sludge combined with a reduction of sludge sent to the landfill (Glowacki 1994).

Proteins are natural polymers, and they have been used for a relatively wide range of applications. At the beginning of the 20th century, proteins were considered interesting raw materials for making plastics to eventually replace cellulose. The first patents were obtained in the 1920s on the use of zein to formulate different materials (coatings, resins, and textile fibres). At that time, formaldehyde was widely used in blends with soybean proteins and slaughterhouse blood to make automotive parts, e.g. distributor caps. In addition, gelatin was used to produce films for foods, drug capsules, and photography. In the 1960s synthetic plastics started to replace the proteins in most applications. In the last three decades, however, there has been a renewal of interest in the development of biodegradable or recyclable materials, mostly for ecological benefits. Currently, biodegradable materials are used in many applications such as agricultural mulching films, wide varieties of packaging materials, and even in medical applications. The proteins, because they are natural polymers, are fully biodegradable (Guilbert and Cuq 2005). Moreover, if an extracellular protein is an enzyme, there is the option for its usage as a reaction catalyst. The investigation of new proteins and obtaining proteins from alternative sources is highly respected. A wider use of biodegradable polymers is also favored due to the fact that crude oil could then be preserved for applications in which no other alternative materials exist. With this in mind, this research is an approach of producing extracellular fungal proteins from various species of *Trichoderma* while using secondary paper mill sludge as a substrate.

EXPERIMENTAL

Materials and Methods

Secondary paper mill sludge from a Quebec paper mill (ABTB Inc.) was used in this study. The sludge was the product of a softwood kraft pulping process (gymnosperm), mainly composed of spruce, pine, and fir. The secondary sludge (ss) was obtained as a product of biological treatment of the primary sludge in the treatment plant. The pH of the sludge was around 8.5, and total solids content was 2.72%. For this study, the secondary sludge, in the form of a water suspension, was manually mixed to assure

uniformity before it was used as a substrate for fungi growth. Each experiment was repeated 5 times.

Initial efforts to cultivate fungi on sludge without the presence of a nutrient solution were not successful; therefore, a basic nutrient medium was added to the sludge. The fungus was then cultivated in a solution that contained 10 grams of homogenized liquid sludge and 125 mL of nutrient medium. The nutrient medium contained 1g of sucrose, 0.1g of yeast extract, 1g of KH_2PO_4, 0.1g of $MgSO_4$, 0.048g of $FeCl_3.6H_2O$, 0.036g of $MnCl_2.7H_2O$, and 0.044g of $ZnSO_4$ in 1 liter of distilled water. The pH of the prepared mineral medium was 5.5. The media with and without sludge for culture growth were prepared in 500 mL Erlenmeyer flasks, closed by cotton stoppers, covered by aluminum foil, and sterilized in autoclave at standard conditions (125°C for 20 minutes). After sterilization, twenty mycelial plugs (5x5 mm) were transferred into the flasks, and the flasks were kept on an orbit shaker at 150 rpm and room temperature for 7 days. After 1 week of cultivation, the fungi were removed from the cultures by simple filtration. The clear filtrate of 30 mL was concentrated to 1 mL by lyophilisation. The control sample containing secondary sludge and nutrient media without the fungus was prepared. The control sample was kept on an orbit shaker for the same time and at the same conditions as fungal cultures, the concentration procedure was also identical. The 7 species of Trichoderma used in this study are listed in Table 1.

Table 1. The Source of Fungal Isolates Used in the Study

No.	Name	Description - origin
1	*Trichoderma harzianum*	ATTC 20476
2	*Trichoderma aureviride*	UTHP 0408 B91 (conf.DNA)
3	*Trichoderma hamatum*	Boreal Mixedwood Forest, ON, CA
4	*Trichoderma viride smooth*	Boreal Mixedwood Forest, ON, CA
5	*Trichoderma virens*	Boreal Mixedwood Forest, ON, CA
6	*Trichoderma polysporum*	Boreal Mixedwood Forest, ON, CA
7	*Trichoderma koningii*	Boreal Mixedwood Forest, ON, CA

Composition analysis of secondary paper mill sludge

The water content in raw untreated secondary sludge was determined by total drying of 250 g of liquid material by freeze drying. The weight difference between sludge before and after drying was confirmed as the water content.

The ethanol/toluene extractable substances such as waxes, fats, resins, and phytosterols were determined according to TAPPI Test method T204 om-88, by measuring of the dry extract weight from 5 hours extraction in Soxhlet apparatus.

The percentage of soluble proteins present in secondary sludge was estimated by using a protein assay kit (BioRad, US). The protein assay is based on Bradford dye-binding procedure (Bradford 1976).

The holocellulose was estimated by the method of Wise (Wise et al. 1946) in ethanol/toluene extractives free sludge sample.

The Klason lignin was determined according to TAPPI Test Method T 222 om-88. Briefly, the ethanol/toluene extractives free sludge was hydrolyzed by 72% sulphuric acid for 2 hours at 20°C. The suspension was diluted with water to 3% acid concentration, then boiled for 4 hours while maintaining constant volume. The vacuum filtration

and washing with water followed. The sample was dried and weighted to determine the amount of Klason lignin. The acid-soluble lignin was quantified by measuring the absorbance of filtrate at 205 nm.

The ash content was estimated by TAPPI Test Method T 211 om-07.

Gel electrophoresis

Gel electrophoresis is a technique that applies an electric field to a gel matrix and used for the separation of protein molecules. Extracellular proteins were separated using SDS-PAGE gel electrophoresis. The gradient concentration of the gel was 4% to 12% (BioRad, US). The prestained SDS-PAGE standards (broad range; 7.1-209 kDa; BioRad, US) were used as markers, and 2-mercaptoethanol was used as a reducing agent to denature the proteins. Electrophoresis was performed using a Bis-Tris buffer system at 150V for an hour or until the blue dye reached the bottom of the gel. Following the electrophoresis, the gel was stained by BioSafe Coomassie Stain (BioRad, US).

FTIR analysis of fungi treated secondary paper mill sludge

Fourier transformed infrared spectroscopy analysis results were recorded on a TENSOR^{TM} Series spectrophotometer (Bruker, USA), on potassium bromide (KBr) pellets. The pellets were prepared from a mixture of a 2 mg sample and 100 mg of KBr. Before the pellets were prepared, the samples and KBr were separately dried at 100°C for 4 hours to avoid possible influence of the moisture on the analysis. The spectra were created using a frequency range of 400 to 4000 cm^{-1}.

RESULTS AND DISCUSSION

Composition Analysis of Secondary Paper Mill Sludge

The results of the composition analysis of the secondary paper mill sludge are listed in Table 2. The water content of the secondary sludge was quantified. The contents of ethanol/toluene soluble materials, water soluble proteins, holocellulose, Klason lignin, acid soluble lignin, and ash in secondary sludge solids were determined.

The analysis proved that used secondary paper mill sludge contained the characteristic wood components. These substances could therefore form a part of the growing substrate for tested wood decay fungi genus of the *Trichoderma*.

Table 2. The Results of Composition Analysis of Secondary Paper Mill Sludge

Secondary sludge component	[weight %]
Water	97.28
Solids	2.72
Tested components of the solid sludge fraction	
Ethanol/toluene soluble materials	8.5
Water soluble proteins	3.7
Holocellulose	35.4
Klason lignin	18.6
Acid soluble lignin	3.8
Ash	23.5

Gel Electrophoresis

Prepared samples of extracellular fungal proteins, produced by different isolates of the fungus *Trichoderma* in nutrient medium/secondary sludge solutions, were separated by gel electrophoresis. The results of the gel electrophoresis (Fig. 1) clearly indicate that all studied species of *Trichoderma* are capable of growth on a secondary paper mill sludge substrate. The production of extracellular proteins from fungi was enhanced by the presence of sludge when compared with cultures without sludge. Higher protein concentrations were expressed by more significant bands determined by the gel electrophoresis in the fungi/secondary sludge samples. Notably, there were detected quantitative differences in extracellular protein production. The visible qualitative differences in band positions were detected in only the *T. hamatum* species, (for example, in Lane C, protein band #13 compare to the sample Lane J). Figure 1 also shows that *T. harzianum* and *T. aureviride* exhibited very similar protein profiles, while the other 5 species of *Trichoderma* each exhibited unique protein profiles.

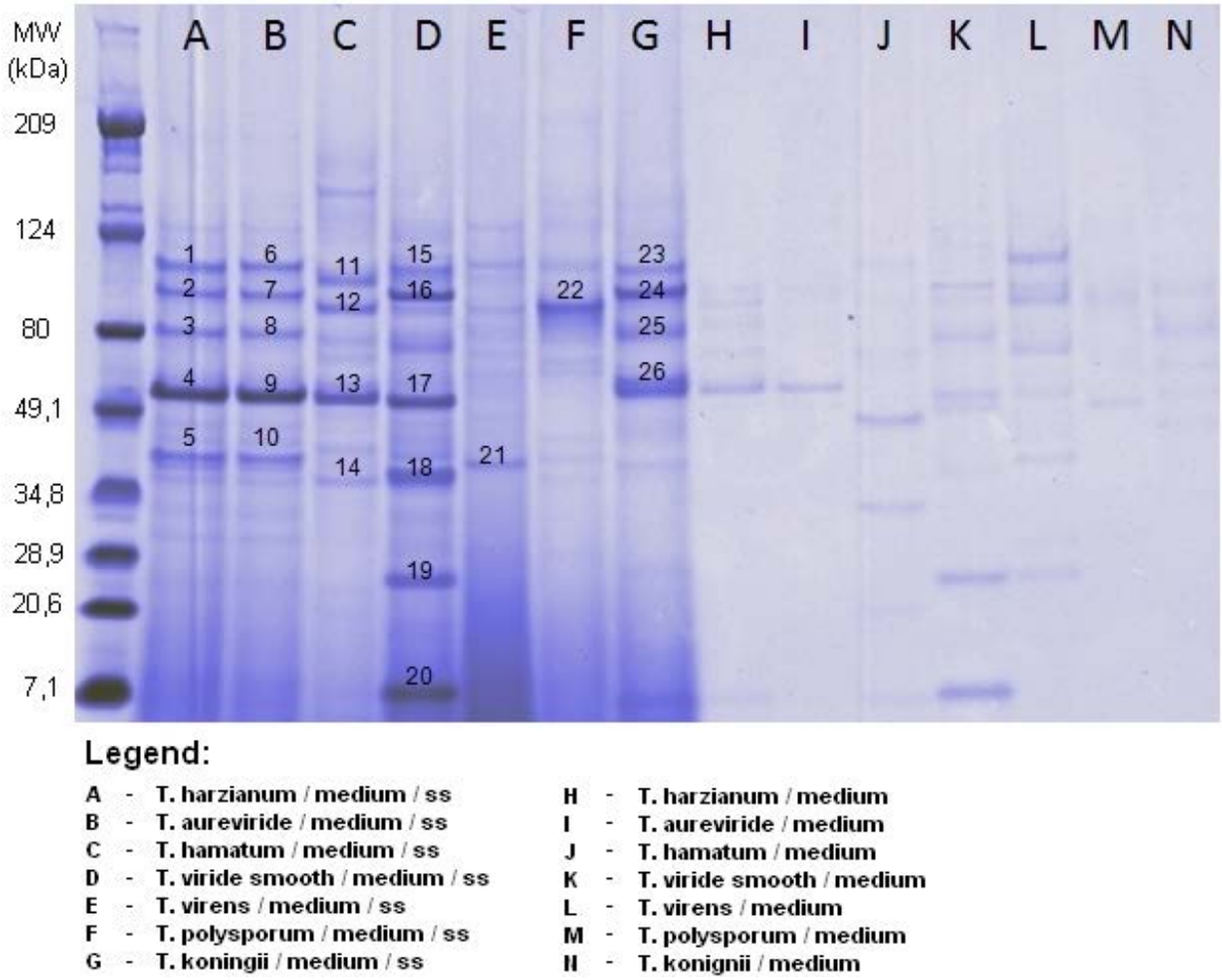

Fig. 1. The extracellular protein patterns of the 7 species of *Trichoderma* grown in nutrient medium with (Lanes A – G) and without (Lanes H – N) secondary paper mill sludge

The gel electrophoresis of culture composed of secondary sludge and nutrient medium, without fungal treatment was performed. No visible protein bands were observed in this sample (data not shown). This result proved that the proteins detected in samples treated by fungi originated from fungal activity on the sludge substrate, not from the secondary sludge itself.

As seen in Fig. 1, these fungi can utilize secondary sludge as a substrate for fungal protein production. According to the intensity of the protein bands, it seems that the *T. harzianum* and *T. aureviride* species produced the highest amounts of the particular protein (Fig. 1, bands 4 and 9). Based on the nature of the proteins, they may have the potential for organic waste treatment.

Future research development should include determining the genome sequence of *T. harzianum*. The purpose of the DNA sequencing is to determine the exact identification of the gene responsible for the generation of the particular protein. Higher production of the particular protein could be achieved by genetic modification of the gene responsible for the generation of the particular required protein; for instance, using cloned bacteria of *Escherichia coli* the desired protein could be produced in large quantities during fermentation.

FTIR Study

Secondary sludge is a waste material composed of wood components such as lignin, cellulose, hemicellulose, and small amounts of mineral ash, lipids, and proteins. It also contains waste water from the pulping process. The ratios between the components varies depending on the type of wood pulped (softwood/gymnosperm or hardwood/ angiosperm) and the pulping method used (kraft process, soda process, or sulfite process).

The sludge used in this experiment originated from a kraft process that pulps mostly softwood. The kraft pulping process uses a mixture of sodium hydroxide and sodium sulfide to break down the bonds that link lignin and cellulose, resulting in pulp that is used for paper production. A large amount of pulp, however, is also found as the main component in primary sludge. The primary sludge is usually further processed biochemically to form secondary sludge. This process not only produces paper mill waste but also has consequences that are complex and environmentally undesirable. In this research, Fourier transform infrared spectroscopy (FTIR) analysis was performed for better understanding the chemical composition of secondary paper mill sludge and its effect on fungal cultivation.

Figure 2 shows the spectra of the untreated secondary sludge (A) and the secondary sludge after cultivation by each of the studied fungi (B-H). The FTIR spectra of the untreated sludge expressed almost all the peak positions present in the spectra of wood samples in previous publications (Pandey and Pitman 2003). The secondary sludge contains major wood components, but these components are found in different ratios and arrangements. The linkages between components were broken down during pulping, freeing most of the cellulose that is carried through the remainder of the process. All the sludge samples used as a substrate for fungi growth (Fig. 2, B-H) resulted in almost identical peak positions as the untreated sludge, but showed more intense bands. Band intensities were also observed to be dependent on the species of *Trichoderma* used.

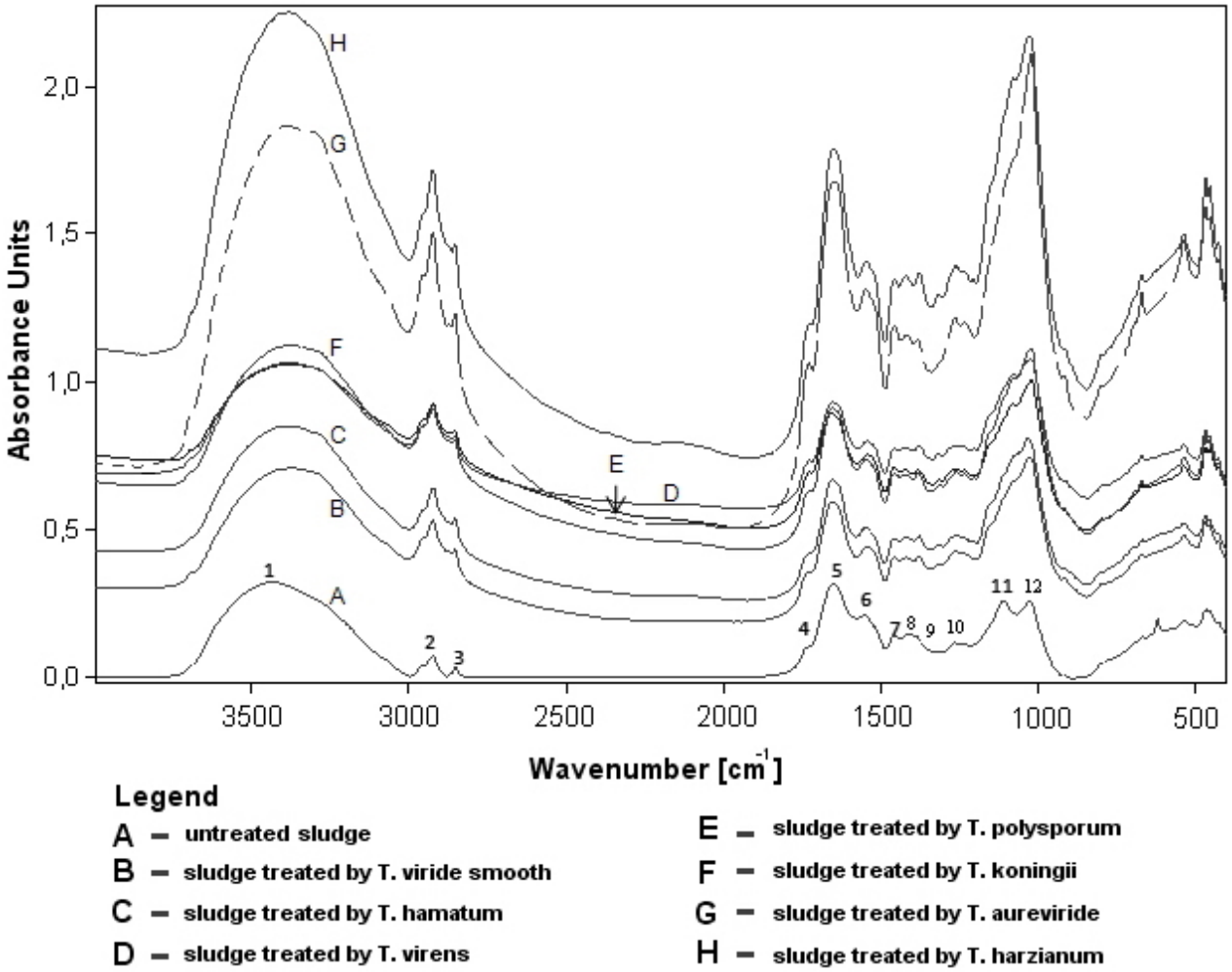

Fig. 2. FTIR analysis of secondary paper mill sludge with and without fungal treatment

According to previous studies (Pandey 1999) and general knowledge of paper mill sludge composition, it is possible to assign observed characteristic peaks to certain chemical groups. Table 3 lists the identified peaks of the FTIR analysis of the untreated sludge and the sludge cultured by *Trichoderma harzianum*. The sludge treated with *T. harzianum* expressed larger absorbances than the untreated sludge, while most of the peak positions were almost identical. At 3345 cm^{-1} (1) there is strong hydrogen bond (O-H) stretching absorption in the spectrum of the untreated secondary sludge. Based on the results of the gel electrophoresis, it is conclusive that the fungal organism produced extracellular proteins. The proteins are composed of amino-acids linked by peptide bonds. Chemically, the peptide bond consists of carbonyl and secondary amine functional groups. The presence of peptide bonds in the samples treated by *T. harzianum* and *T. aureviride* contributed to an increase in the absorbance in the range of 3000 cm^{-1} to ca. 3600 cm^{-1}, within which the carbonyl and secondary amine groups absorbed the infrared light (Fig. 2). The differences in the FTIR analysis results of the untreated sludge and the sludges tested with *Trichoderma* are not as significant. The prominent C-H stretching

absorption occurs at 2915 (2) and 2848 cm^{-1} (3) in the spectra of the untreated sludge. The peak in the fingerprint region (between 1800 and 600 cm^{-1}) at 1740 cm^{-1} (4) is credited to (Pandey 1999) unconjugated C = O in the xylans (hemicellulose). In the sludge treated by *T. harzianum* this band developed at 1724 cm^{-1} and the intensity of the band was greater than that of the untreated sludge. The band at 1661 cm^{-1} (5) represents the O-H bonds, while the conjugated C-O bonds are manifested at 1554 cm^{-1} (6). The aromatic lignin skeletal structure is identified by the peak at 1459 (7). The peak at 1401 cm^{-1} (8) represents C-H deformation in the lignin and carbohydrates. The band at 1258 cm^{-1} (10) is most likely associated with C-O of guaiacyl ring breathing, C-O stretching in lignin, or C-O linkage in guaiacyl aromatic methoxyl groups.

Table 3. Summary of Identified FTIR Bands Observed in Untreated Secondary Sludge (SS) and Sludge used for *Trichoderma harzianum* Cultivation

Peak No.	SS without treatment	SS treated by *T. harzianum*	Assignment
	Band [cm^{-1}]	Band [cm^{-1}]	
1	3445	3390	O-H stretch (hydrogen bonded)
2	2915	2928	C-H stretching absorption
3	2848	2852	C-H stretching absorption
4	1740	1724	unconjugated C=O in xylans (hemicellulose)
5	1661	1643	absorbed O-H, conjugated C-O
6	1554	1547	conjugated C-O
7	1459	1451	aromatic skeletal in lignin
8	1401	1414	C-H deformation (asymmetric) in lignin and carbohydrates
9	Shoulder of 1401	1366	C-H deformation (symmetric) in cellulose and hemicellulose
10	1258	1258	C-O of guaiacyl ring breathing
11	1110	1077	Guaiacyl C-H and syringyl C-H, or aromatic skeletal and C-O stretch in cellulose and hemicelluloses, C-O of secondary alcohol
12	1020	1029	C-O stretch in cellulose and hemicellulose, C-O of primary alcohol, guaiacyl C-H
Source of the assignments in Table 3: (Pandey and Pitman 2003), (Pandey 1999)			

This experiment revealed that fungal cultivation on a secondary sludge substrate leads to substrate enrichment by the products of the fungal metabolism. The presence of additional substances and the absence of fungi-consumed sludge components resulted in higher intensities of particular bands by FTIR analysis. Very similar results were previously recorded using wood samples exposed to brown rot and white rot fungi for various durations of time (Pandey and Pitman 2003). The study found that an increase in the time allowed for wood decay resulted in higher intensities of particular bands in FTIR results. In the current research, the duration and conditions of the cultivation were held constant, but variations in the band intensities depended on the particular species of *Trichoderma*. This phenomenon is a result of specific enzymatic activity of each tested

fungal species. The most significant band intensity increase was found using the sludge treated with *Trichoderma harzianum* and *T. aureviride*. With this particular sludge as a substrate, *T. harzianum* seemed to be the best candidate for cultivation due to its fast growth and high production of the extracellular protein in comparison with other *Trichoderma* tested. The mycelial growth of *T. harzianum* was visible with the naked eye after only 24 hours in the shaking culture.

The peaks at 1110 cm^{-1} and 1020 cm^{-1} (Fig. 3) are probably representative of primary and secondary alcohols in the sample of untreated secondary sludge. Without cultivation there was a peak at 1110 cm^{-1}, which was then found at 1077 cm^{-1} with lower intensity in sample cultured by *T. harzianum*; however, the intensity of this band could have also been suppressed by the strong absorption at 1029 cm^{-1}. It is apparent that the digestion of the original sludge components by fungal organism caused the observed changes in the chemical arrangement of the substrate.

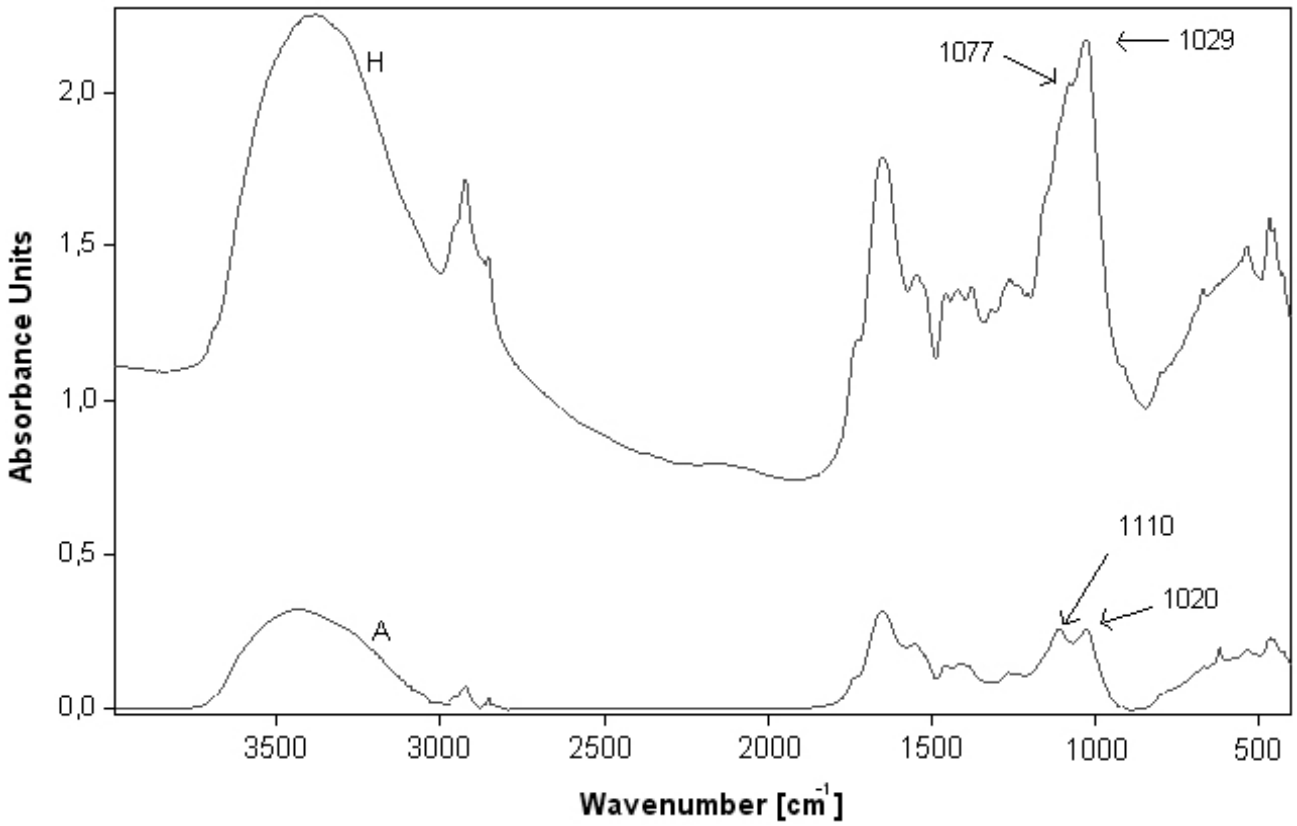

Fig. 3. FTIR results of the untreated sludge sample (A) and sample treated by *T. harzianum* (H)

Currently, the residues of chemicals commonly used during wood delignification cause a serious environmental problem; however, there is no conclusive data to suggest that the fungi decompose only wood components or harmful chemicals. This waste material should be treated as complex, because the separation of polluting chemicals from wood components does not have a simple and low-cost solution.

CONCLUSIONS

1. Secondary paper mill sludge has great potential to function as a valuable carbon source for fungi growth, especially in the case of various *Trichoderma* species. The results of this research clearly demonstrate that these fungi are capable of growth while using the studied secondary paper mill sludge as a substrate. The fungal growth, however, must be supported by the presence of mineral medium in the fungal cultures.
2. In the presence of secondary sludge, all the tested fungi species showed an increase of extracellular protein production, mostly of the same molecular weight as those produced in cultures without secondary sludge. For an exact description of the extracellular proteins produced by the *T. harzianum* fungi, further analysis is necessary to learn of its genomic DNA sequence.
3. Based on the FTIR spectra analysis of the untreated secondary sludge samples, the samples maintained practically most of the functional groups that are found in original wood samples. These functional groups were identified by the characteristic absorption peaks for cellulose, lignin, and hemicellulose in the FTIR spectra.
4. Secondary sludge used as a fungal culture achieved higher intensities of particular FTIR bands, compared with that of pure sludge. This effect was caused by the fungal activity. Generally, the fungal activity included consumption of specific sludge components, growth of the mycelium, and production of extracellular proteins and other metabolites. Accordingly, the fungal organism consumed some sludge substrate components for growth, simultaneously enriching the substrate with by-products of its metabolism.
5. Using secondary sludge as cultivation substrate, the *T. harzianum* and *T. aureviride* fungi were determined to be the most promising fungal organisms of the species tested. This conclusion was based on the fact that the *T. harzianum* and *T. aureviride* fungi showed the best growing capability in the shaking cultures.

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