Potential Application of *Ganoderma lucidum* in Solid State Fermentation of Primary Sludge and Wheat Straw

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This study was conducted to investigate the production of lignocellulolytic enzymes and sugars by the fungus *Ganoderma lucidum* during solid state fermentation (SSF) using primary sludge (PS) and wheat straw (WS) as substrates at different concentration ratios. For fungal growth on SSF, 20 g of each blended substrate was added to Erlenmeyer flasks, which were autoclaved and maintained at room temperature prior to inoculation, whereas for submerged fermentation (SF), flasks containing 25 mL of potato dextrose broth (PDB) were used as standard to check the differences between both methods of growth, and then all flasks were incubated at 25 °C in the dark, during 8 and 16 days. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis from the protein extract obtained from solid state fermentation strongly suggested that *G. lucidum* could produce lignocellulolytic enzymes to degrade primary sludge and wheat straw. Among the sugars, the production of xylose and mannose was disturbed by adding primary sludge. With the addition of primary sludge, high glucuronic acid content was observed. The results suggest that the combination of primary sludge and wheat straw, at concentration ratios of 1:1 to 1:3, respectively, can be used as a raw material in the production of lignocellulolytic enzymes and the bioconversion of other types of biomass by *G. lucidum.*

Keywords: Ganoderma lucidum; Solid state fermentation; Lignocellulolytic enzymes

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INTRODUCTION

The pulp and paper industry plays an important role in the global economy. However, the dumping of bio-solids, known as sludge, is the primary concern, where large amounts of sludge from the pulp and paper companies are accumulated on site. This waste can cause serious handling and pollution problems, especially under an increasingly tight economic situation and strong environmental protection policies (Rashid *et al*. 2006). The pulp and paper industry generates between 4 to 5 million tons of sludge per year in the USA (Fan and Lynd 2007a,b). In Japan, about 5 million tons of paper sludge is produced and discarded annually by the paper industry (Kang *et al*. 2010), while most of the 7.1 Mt/a of dry sludge produced in 1995 in Canada was disposed of

through combustion and landfilling (Beauchamp *et al.* 2002). Industrial wastes from crop cultivation and food processing constitute a vast available renewable resource for microbial conversion into different value-added products.

In this scenario, white rot fungi have suitable potential to grow on lignocellulosic biomass due to their ability to produce a variety of hydrolytic and oxidative enzymes (Mtui 2012). There are promising developments in the application of solid-state fermentation (SSF) in enzymatic bioprocessing of lignocellulosic biomass. Due to the ability to produce lignocellulolytic extracellular enzymes, white rot fungi are the main degraders of lignin constituents of wood, and therefore they are a potential asset to bio-pulping processes (Singh *et al.* 2011).

The methods reported to degrade lignocellulose include chemical, physical, mechanical, and biological degradation (Isroi *et al.* 2011), among which, biological degradation, such as SSF, is known to be cheaper, safer, less energy consuming, and more environmentally friendly (Salvachúa *et al.* 2011). The non-specificity of the ligninolytic enzymes used by many white rot fungi species for the substrate degradation may explain their ability to biodegrade a wide spectrum of organic environmental pollutants (Nandakumar *et al.* 1994; Rajarathnam *et al.* 2001; Membrillo *et al.* 2008).

Enzymes have a substantial role in the production of a variety of commercial products for many bio-technological purposes (Gavrilescu and Chisti 2005; Kuhad and Singh 2007). These enzymes not only make the process environmentally friendly, but also play an important role in improving productivity, eventually lowering the cost of the final product. For the last two decades, among various industrial enzymes, cellulases, xylanases, and laccases have gained an enormous amount of attention for their potential applications in the bioconversion of biomass and other biotechnological applications. These enzymes have been used in such applications as bio-stoning and bio-polishing of jeans, improving efficacy of detergents, retting of flax, bio-pulping, treatment of wastewater polluted by dyes and other organic pollutants, development of biosensors, improving nutritional properties of animal feed, maceration and color extraction from juices, and the production of oligosaccharides (Xu 2005; Kuhad and Singh 2007; Kuhad *et al.* 2010).

Mushrooms have the ability to degrade lignocellulosic substrates such as unfermented agricultural wastes, woodland residues, animal husbandry residues, and manufacturing residues through SSF (Moldes *et al*. 2003; Rodriquez-Couto and Sanroman 2005; Shah *et al.* 2005; Stajic *et al.* 2006; Pant *et al.* 2006; Pant and Adholeya 2007; Sánchez 2009). One of the most common raw materials for mushroom cultivation, wheat straw (or bran), represents a very common agricultural residue worldwide, and it contains soluble carbohydrates and inducers of enzyme synthesis (Kim *et al.* 2006; Murugesan *et al.* 2007; Li *et al*. 2007; Stajić *et al.* 2010). Paper sludge, when disposed of in the environment, can create serious degradation problems; however, this kind of waste contains sufficient nutrients for microbial growth that are a potentially attractive raw material for production of fermentation products such as ethanol *via* SSF or enzymatic hydrolysis (Katzen and Fowler 1994; Duff *et al.* 1995; Jeffries and Schartman 1999; Lark *et al.* 1997; Lynd *et al.* 2001; Fan *et al.* 2003).

In the present study, we studied the production of lignocellulolytic enzymes produced under conditions of solid state fermentation that were capable of degrading primary sludge with or without wheat straw, which may have implications for future biotechnological application.

EXPERIMENTAL

Materials

Fungus

The basidiomycete fungus *Ganoderma lucidum* UAMH 8026 was purchased from the University of Alberta Microfungus Collection and Herbarium (Edmonton, Alberta, Canada). The fungal culture was maintained on a commercial potato dextrose agar (PDA) at 24 ± 1 °C, transferred by periodical subculturing, and stored at 4 ± 1 °C.

Raw materials and biomass preparation

Primary sludge (PS) was obtained from Abitibi Bowater Inc. (Ontario, Canada), and the wheat straw (WS) was obtained from a local grower in Guelph (Ontario, Canada). The primary sludge was freeze dried with a Vis Tri Freezemobile 35 Freeze Dryer (SP Industries Inc., Warminster, PA) at -80 °C. Since the fresh materials had a high moisture content (above 65%), the WS and freeze-dried PS were mechanically cut into small pieces by a chopper, as described by other investigators (Kuhad *et al.* 2010; Gupta *et al.* 2011), and stored in sealed plastic bags at room temperature. The moisture content for each substrate was adjusted to 62 ± 2% (v/w) prior to use. The individual and blended substrates prepared for the study are listed in Table 1.

For fungal growth and protein profile on Solid State Fermentation (SSF) 20 g of each blended substrate was added to a 250 mL Erlenmeyer flask, sealed with cotton wrapped in gauze, autoclaved for 60 min at 121°C (1.5 atm), and maintained at room temperature prior to inoculation. For Submerged Fermentation (SF), flasks containing 25 mL of potato dextrose broth (PDB) was surface inoculated with five PDA disks (6 mm diameter) containing *G. lucidum* mycelium from five day old cultures. All flasks were incubated at 25 °C in the dark, tapped with cotton plugs and plastic film to prevent evaporation, during 8 and 16 days.

Table 1. Experimental Design: Preparation of Individual and Blended Substrates

Treatment	Substrates (ratio)	Substrates Weight			Water[d]	Moisture
		Dry[a] (g)	Fresh[b] (g)	Total[c] (g)	mL	%
T1	PS / WS (1:1)	1.3 / 1.3	1.7 / 1.3	7.0	4.0	60.6
T2	PS / WS (3:1)	1.9 / 0.6	2.5 / 0.7	7.6	4.5	64.9
T3	PDB	15.0	-	300.0	315.0	99.0
T4	PS	2.5	3.3	7.8	4.5	64.3
T5	WS	2.5	2.7	6.2	3.5	63.4

PS**: primary sludge; WS: wheat straw; PDB: potato dextrose broth** [a]Total weight after freeze-dry; [b]Total weight after storage, prior to use; [c]Fresh weight plus water; [d]**Distilled water** Added to fresh substrate

Methods

Submerged fermentation

To check the protein profile, SF using PDB was chosen as the standard. Fungal growth was carried out in 125 mL Erlenmeyer flasks containing 30 mL of medium, which was sterilized at 121 °C for 15 min and maintained at 25 °C prior to inoculation. Five PDA disks (6 mm diameter) containing *G. lucidum* mycelium from five-day-old cultures were transferred to each flask and incubated on a rotary shaker at 150 rpm, 25 ±

1 °C. Submerged fermentation occurred for over 16 days, with protein profile, soluble carbohydrate content, and biomass collected and analyzed on the eighth and 16th day.

Solid state fermentation

SSF was carried out in an incubator at 24 °C, in 125-mL Erlenmeyer flasks containing 2.5 g of the lignocellulosic substrates. The moisture was obtained by adding 4 mL of double distilled water in order to adjust to 62 ± 2%. The initial pH of the substrates was adjusted to 5.5 prior to sterilization by adding $CaCO_3$. The substrates were sterilized at 121 °C for 15 min and maintained at 25 °C prior to inoculation. Each flask was inoculated with five 6-mm PDA disks containing mycelium pre-cultivated for five days at 25 °C. Solid-state fermentation proceeded for 16 days, with protein profile, soluble carbohydrate content, and biomass collected and analyzed on eighth and sixteenth days.

Weight loss

After solid and liquid fermentation, the substrates were weighted to check the loss during mycelial growth.

Organic matter content

The loss-on-ignition (LOI) method, often referred to as ashing, is a simple and relatively inexpensive method widely used in soil science for determining organic matter (Nelson and Sommers 1996; Konen *et al.* 2002). Samples from each flask (0.5 g) cultivated through SSF were analyzed for determination of ash content involving incineration in a muffle furnace (Gallen Kamp hot box) at 550 °C for 2 h according to the *Test Methods for the Examination of Composting and Compost* (TMECC) (Thompson *et al.* 2002).

Protein profile using the SDS-polyacrylamide gel electrophoresis (PAGE)

For SF, the biomass from mycelium plugs was transferred to the flasks containing PDB; for SSF, mycelium plugs were carefully covered with the different substrates using a sterile spatula. After different incubation periods (standard, 8 and 16 d): the flasks containing PDB cultivated through SF, solids were separated by filtration using a Millipore filter (0.22 µm) into 50 mL centrifuge tubes; for the substrates PS and WS cultivated through SSF, the content of each flask was suspended in 30 mL of a 4 mM sodium bicarbonate solution (pH 8.9) for 12h and solids were separated by filtration and stocked in 50 mL centrifuge tubes. Clean protein extract was freeze-dried with a Vis Tri Freeze Dry at -80 °C. The protein extract was dissolved in distilled water at a saturated concentration prior to electrophoresis. A protein extract sample (duplicate) was prepared by adding 6.5 µL of the saturated protein solution with 2.5 µL of 4x NuPAGE LDS sample buffer (Invitrogen, USA) and 1 µL of 10X NuPAGE Reducing Agent (or β-Mercaptoethanol), making a total volume of 10 µL, according to the manufacturer's recommendations.

For SDS-PAGE analysis, 25 µL of each protein extract sample, along with the broad range molecular marker (Bio-Rad), was loaded onto a NuPAGE Novex Bis-Tris 4-12% gel in a Novex XCELL mini electrophoresis apparatus, according to supplier instruction. Electrophoresis was performed at a constant voltage (200 V) for about 50 min. After electrophoresis, the gel was removed and stained overnight with a 0.04% of Coomassie blue solution. The gel was washed with a de-staining solution until the background was clear.

Soluble carbohydrate

For determination of sugars (*i.e.,* arabinose, galactose, glucose, xylose, and mannose) and glucuronic acid, samples of 3 g from each substrate (duplicate), before and after fungal growth, were acidified with one volume 10 wt% sulfuric acid per nine volumes sample and centrifuged at 1500 rpm for 15 min. The supernatant was transferred to a 100-mL serum bottle and autoclaved sealed at 121 °C for 1h. After cooling, the supernatant was analyzed using an Aminex HPX-87H column (Bio-Rad, Foster City, CA) *via* a Dionex DX-600 HPLC (high pressure liquid chromatography) system equipped with an UV detector (Dionex Corp.; USA) similarly as described previously (Lynd *et al.*, 2001).

Experimental design

The experimental design was a completely randomized factorial of 5x3, corresponding to five substrates and three stages of colonization. Five replicates (duplicates for 8^{th} and 16^{th} day and one flask for day 0 as standard) were prepared for each substrate, with non-inoculated flasks prepared as standards totaling 25 flasks. Each replicate corresponded to a 125-mL Erlenmeyer flask containing 7.0 of fresh lignocellulosic substrate for solid state fermentation (totaling 20 flasks) and 30 mL of PDB for submerged fermentation (5 flasks). The data were subjected to analysis of variance in duplicates. Means were compared by Tukey's range test, at a confidence level of 95%, using the statistical program ASSISTAT 7.5 Beta developed by Dr. Francisco de Assis in the Department of Agricultural Engineering, Center for Technology and Natural Resources, Federal University of Campina Grande (UFCG), Brazil (Silva and Azevedo 2002).

RESULTS AND DISCUSSION

Biological degradation of sludge and wheat straw is a complex process affected by many factors, and analysis of optimum growth conditions for obtaining the maximum amount of fermentable sugars is the first step to understand the mechanisms of fungal degradation (Guillén *et al.* 2000; Wan and Li 2010). The results obtained clearly showed that the fungus *G. lucidum* is capable of producing lignocellulolytic enzymes during growth in primary sludge mixed with wheat straw. Also, we observed that *G. lucidum* range from faster to slower mycelial growth according to the amount of raw material ratio used.

As expected, the best substrate for mycelial growth was wheat straw as noted by Stajic *et al.* (2010). However, when the substrates were mixed, optimal mycelial growth rates of *G. lucidum* relative to the concentration ratio of primary sludge and wheat straw (PS:WS) was found in WS, followed by PS:WS at a ratio of 1:1 and 1:3, and finally in PS (Fig. 1).

Since the PDB culture is a liquid medium, it was not possible to determine the degree of colonization. The typical parameters for solid substrates, such as mycelial vigor and degree of colonization, were not relevant in the liquid cultures since the flasks were stirred and the mycelium formed pellets rather than branches (Fig. 1H). Due to this, the pellets concentrated biomass from the fungal culture indicating bioconversion.

The dry weight of the substrate protein extract can be used as an indicator for the metabolic activities of *G. lucidum* in the various substrates. It can be seen that the dry weight of the extracts on the first day of fungal growth was higher in the PDB (Table 2).

This represented the total content of dry material in that volume of the PDB. However, after eight days of fungal growth, except for PDB, the dry weight of all other medium increased, showing statistical differences among the media. The F value for the substrate was 12.1828, significant at 1% level (Tukey), due to fact the media possess different compositions and the F-value for the interaction (substrates X days) was 53.4270, significant at 1% (Table 5), clearly suggesting that the production of proteins did accumulate during the growth period and differ statistically.

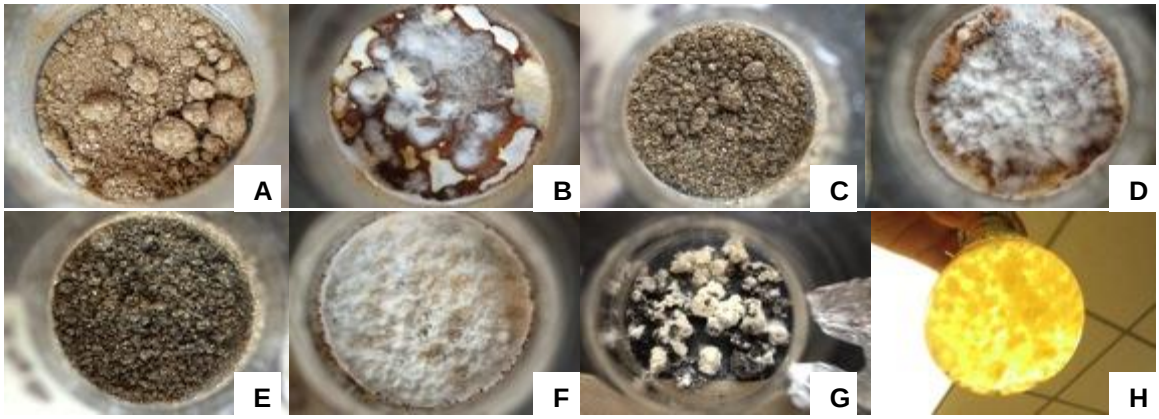

Fig. 1. Substrates before and after 16 days of solid state fermentation by *G. lucidum* UAMH 8026. A: Wheat straw before and (B) after fungal growth; C: Primary sludge and wheat straw, 1:1, before and (D) after fungal growth; E: Primary sludge and wheat straw, 3:1, before and (F) after fungal growth; G: Primary sludge after fungal growth; H: Potato-dextrose-broth after fungal growth

Table 2. Protein Extract Dry Weight (g) (mean ± sd) of Samples after 0 and 8 Days of Growth of *Ganoderma lucidum* on Substrates Based on Single Primary Sludge and Wheat Straw

Substrate (g)	Days			
	0		8	
PDB	0.082 ± 0.016	**aA**	0.018 ± 0.003	**bB**
WS	0.038 ± 0.005	**bA**	0.048 ± 0.007	**aA**
PS:WS (1:1)	0.019 ± 0.003	**cB**	0.037 ± 0.005	**aA**
PS:WS (3:1)	0.026 ± 0.004	**bcB**	0.044 ± 0.006	**aA**

PDB = Potato Dextrose Broth; WS = Wheat Straw;
Lower case letters compare means between the substrates on the day of growth across substrates; Upper case letters compare means between the days of growth within the same substrate
Tukey's test at confidence interval 95%

As the weight of the PDB protein extract decreased as it was consumed during mycelia growth, the proteins may have attached to the fungal cell walls. Another factor that may explain the fungal growth in the substrates based on primary sludge is an increase of organic matter intake. These substrates showed higher consumption compared to wheat straw, differing statically, suggesting that the PS:WS at ratios of 1:1 and 1:3 favored the growth of the fungi (Tables 3 and 5). The nutrients in the blended substrate provide greater adaptability and production potential of the enzyme on the substrate, compared to the use of each waste separately.

Table 3. Organic Matter (g) of Samples After 0 and 8 Days of Growth of *Ganoderma lucidum* on Substrates Based on Single Primary Sludge and Wheat Straw

Substrate	Days	
	0	8
PS	0.33 dA	0.29 dB
WS	0.48 aA	0.47 aA
PS:WS (1:1)	0.38 cA	0.39 cB
PS: WS (3:1)	0.41 bA	0.37 bB
PS = Primary sludge; WS = Wheat straw		

For the sugar consumption, there was a significant statistical difference only between the substrate interaction and the days of growth for the mannose, with F-value of 17.1143 significant at 1% level on Tukey test, where it can be observed as higher consumption of this sugar only for the substrate based on wheat straw (Tables 4 and 5). Perhaps the sugar degradation can only be seen after more days of mycelial growth.

Table 4. Sugar and Glucuronic Acid Content of the Samples after 0 and 8 days of Growth of *Ganoderma lucidum* on Substrates based on Single Primary Sludge and Wheat Straw

Substrates ratios	Sugars content (ppm)			
	Arabinose			
	0		8 days	
WS	16.2	**a**	15.3	**a**
3:1	8.7	**b**	8.7	**b**
1:1	9.2	**b**	7.5	**b**
	Galactose			
WS	4.7	**a**	6.2	**a**
3:1	6.9	**a**	6.7	**a**
1:1	5.9	**a**	5.1	**a**
	Glucose			
WS	220.6	**a**	192.7	**a**
3:1	71.7	**b**	78.3	**b**
1:1	69.9	**b**	50.7	**b**
	Xylose			
WS	99.7	**a**	91.4	**a**
3:1	20.7	**c**	26.7	**c**
1:1	51.7	**b**	42.1	**b**
	Mannose			
WS	321.0	**aA**	260.3	**aB**
3:1	9.6	**bA**	11.2	**bA**
1:1	7.8	**bA**	7.8	**bA**
	Glucuronic acid			
WS	0	**c**	0	**c**
3:1	266.3	**b**	285.7	**a**
1:1	252.2	**b**	280.6	**a**
WS = Wheat straw Lower case letters compare means between the substrates in the day of growth; Upper case letters compare means within substrates across the day of growth Tukey's test at confidence interval 95%				

Table 5. F-Values Obtained from Analysis of Protein Extract Dry Weight, Organic Matter, Sugars, and Glucuronic Acid Content of the Samples after 0 and 8 Days of Growth of *Ganoderma lucidum* on Substrates Based on Single Primary Sludge and Wheat Straw

Analyzed variables	Variation factor		
	Substrate (S)	Days (D)	S x D
Protein extract dry weight	12.18 **	2.61 ns	53.43 **
Organic matter	11082.55**	551.20 **	96.50 **
Arabinose	25.60 **	0.74 ns	0.26 ns
Galactose	4.99 ns	0.15 ns	2.89 ns
Glucose	88.68 **	1.87 ns	1.10 ns
Xylose	240.14 **	2.11ns	3.34 ns
Mannose	1433.94 **	15.78 **	17.11 **
Glucoronic acid	1457.66 **	11.28 *	3.12 ns

Figure 4 shows the electrophoretic protein profile of the fermentation extract by *G. lucidium* at both 0 and 8 days. While the production of protein occurred during fungal growth, this protein profile was not observed in the fermentation extracts without the fungus (control). This also suggests that *G. lucidum* produced enzymes and degraded these substrates, as reflected in the difference in protein profiles between the extract of the PDB and the PS/WS substrates. The nature of these protein bands appear in the PDB and PS/WS fractions, which are being investigated.

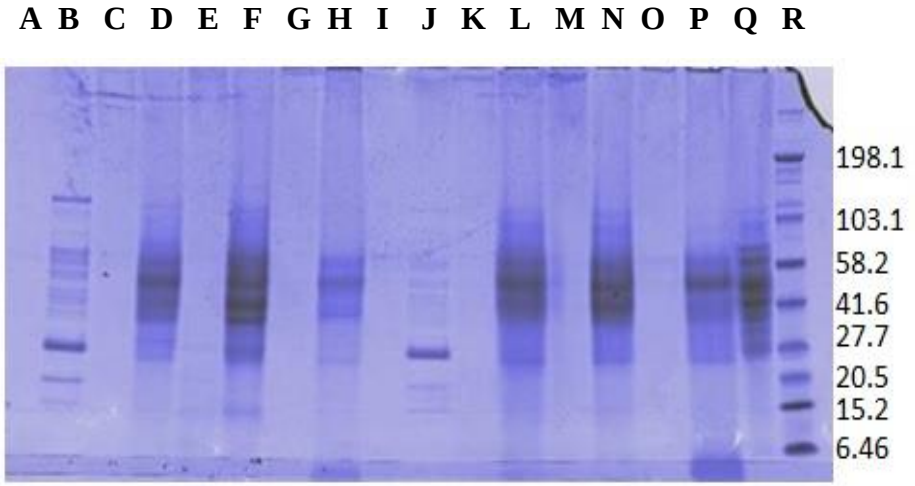

Fig. 4. SDS-PAGE protein profile of *Ganoderma lucidum* cultivated on primary sludge and/or wheat straw without growth (WG) after 8 (8D) and 16 (16D). A and I: PDB, WG; B: PDB 8D; C and K: PS50%/WS50% ; D: PS50%/WS50% 8D; E and M: PS75%:WS25% STD; F: PS75%:WS25% 8D; G and O: WS STD; H: WS 8D; J: PDB 16D; L: PS50%/WS50% 16D; N: PS75%/WS25% 16D; P: WS 16D; Q: PS 16D; R: Broad range marker (KDa)

Apart from wheat bran, other agricultural wastes also can stimulate laccase biosynthesis, and many studies have indicated that fruit peels can be added to culture media as lignocellulosic substrates to enhance the production of laccase (Lorenzo *et al.* 2002; Elisashvili *et al.* 2006; Rosales *et al.* 2007). Like wheat bran, fruit peels contain high levels of sugars, cellulose, protein, and lignin (Grohmann *et al.* 1995), which can

maintain the growth of *G. lucidum*. Many fungal laccase enzymes have been purified and their molecular properties studied. Fungal laccases have a number of different isoforms with a molecular mass ranging from 40 to 80 kDa (Eggert *et al.* 1996; Wang and Li 2010), and this variation could be attributed to the different ecological origins of each species or substrate conditions. In the conditions of work using primary sludge and wheat straw, the major enzymes produced ranged from 40 to 60 kDa. It has been reported that *G. lucidum* produces two isoforms of 40 and 68 kDa (D'Souza *et al.* 1999); however, the molecular weight of laccase was determined to be 43 kDa by other investigators (Murugesan *et al.* 2007). It can be noted as well that the production of lignocellulolytics enzymes was stronger in higher concentration ratio of primary sludge (PS:WS) as the higher 3:1 in the 8th day and 3:1 followed by 1:1 for the 16th day (Fig. 4).

The present work shows that *G. lucidum* is capable of growing on paper sludge and producing specific enzymes for bioconversion of biomass. Bioconversion of cellulosic materials into fermentable sugars is a biorefining area that has invested enormous research effort, as it is a prerequisite for the subsequent production of bioenergy (Kumar *et al.* 2008). For processing cellulosic biomass using commercial cellulase, the cost of production or purchase of enzymes is seen as a major obstacle to cost competitiveness (Vonsivers and Zacchi 1995; Gregg and Saddler 1997; Sheehan and Himmel 1999; Lynd *et al.* 2001; Fan and Lynd 2007a; 2007b). Although recent advances have reduced the cost of production of cellulase, it is still a significant cost component of pulp production at about $0.50 per gallon according to Novozymes (Green Car Congress 2010). According to Lynd *et al.* (2001) and Prasetyo *et al.* (2011), the processing of pulp and paper sludge is a potential entry point and provides support for emerging industrial processes using enzymatic hydrolysis; therefore, the production of value byproducts such as ethanol, the combined profit of ethanol, and the avoidance of dropping the recovery of minerals compared to simple disposal as waste sludge could potentially exceed $200/ton of dry paper sludge.

CONCLUSIONS

1. *G. lucidum* can degrade primary sludge mixed with wheat straw. The mycelial growth rates ranged from faster to slower depending on the concentration ratio of primary sludge and wheat straw, according the following sequence: WS>PS:WS at a ratio of 1:1> PS:WS at a ratio of 1:3>PS. These data strongly suggested that the production of lignocellulolytic enzymes by *G. lucidum* was involved in the fermentation process.
2. The production of extracellular proteins was stronger in higher concentration ratios of primary sludge (PS:WS), as the higher 3:1 in the 8th day and 3:1 followed by 1:1 for the 16th day.
3. In the conditions of work using primary sludge and wheat straw, the major extracellular proteins produced ranged from 40 to 60 kDa.

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