



## POTENCY OF *Trichoderma aureoviride* UPM 09 AND *Fusarium equiseti* UPM 09 IN THE PRETREATMENT AND HYDROLYSIS OF LIGNOCELLULOLOSIC BIOMASS

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### ABSTRACT

**Two new strains of fungi, namely, *Trichoderma aureoviride* UPM 09 and *Fusarium equiseti* UPM 09 were isolated from elephant dung and identified morphologically and through the use of molecular assay. Their genomic DNA was extracted using Epicenter kit<sup>®</sup>. PCR amplification of their genomic DNA was successfully conducted with BIOMETRA Tpersonal/Tprofessional Thermocycler (Germany) using ITS-1 forward primer (5' TCC GTA GGT GAA CCT GCG G3') and the ITS-4 reverse primer (5' GCT GCG TTC TTC TTG ATC GAT GC 3'). The sequences of the fungal strains were deposited in the NCBI (USA) Gen Bank Database and were assigned accession numbers (in parenthesis) and were identified as *Trichoderma aureoviride* strain UPM 09 (JN811063) and *Fusarium equiseti* strain UPM 09 (JN811061). The two fungal strains individually and in consortium were then used for the pretreatment of rice husk (RH), rubber wood saw dust (RW) and oil palm empty fruit bunch (EFB) using solid state cultivation (SSC) and submerged cultivation (SMC). The amount of glucose, reducing sugars and protein from the pretreated lignocellulose biomass was determined using glucose analyzer, DNS reagent and Biorad assay, respectively. The result of this study, therefore, shows that native fungi possess potentials for use in the pretreatment of lignocelluloses biomass. Key Words: pretreatment, lignocellulose, biomass, fungi, cultivation, reducing sugar**

### INTRODUCTION

The major component of biomass is lignocellulose (Pérez *et al.*, 2000) comprising around half of the plant matter produced by photosynthesis (also called photomass) and It represents the most abundant renewable organic resource in soil. It consists of three types of polymers, cellulose, hemicelluloses and lignin that are strongly intermeshed and chemically bonded by non-covalent forces and by covalent cross-linkages. However, only a small amount of the cellulose, hemicelluloses and lignin produced as by-products in agriculture or forestry is used, the rest being considered waste. Many microorganisms are capable of degrading and utilizing cellulose and hemicelluloses as carbon and energy sources (Sanchez, 2009) and useful for pretreatment of biomass (Keller *et al.*, 2003). In this study, two fungal strains isolated from elephant dung, individually and in consortium, were then used for the pretreatment of rice husk (RH), rubber wood saw dust (RW) and oil palm empty fruit bunch (EFB) for hydrolysis of the pretreated samples. The aim of this work is to use the newly isolated fungal strains in the pretreatment and hydrolysis of RH, RW and EFB. The objective of this work is to study the potentials of the fungal strains for their ability to enzymatically hydrolyze the biomass substrates to produce glucose for fermentation as potentials for biofuels production.

### MATERIALS AND METHODS

#### Preparation of Elephant Dung

One gram of the elephant dung was weighed and suspended in a sterile test tube with 10 milliliters of sterile distilled water as slurry before serial dilution was carried out as described by Tripathi (2006). One milliliter (1ml) of the diluted slurry was transferred into a sterile test tube containing 9ml of sterile distilled water. One milliliter of the diluted slurry from the first test tube was transferred into the second test tube containing 9ml of sterile distilled water and was shaken well. The same transfer was done from the second test tube to the third one up to the 9<sup>th</sup> test tube.

#### Inoculation of Fungi

Using streak plate method, one loopful of the dung suspension was inoculated using an inoculating loop on Potato Dextrose Agar medium and incubated at 30°C in an incubator for 4 to 7 days. Colonies that grew were the subcultured to obtain pure colonies.

#### Morphological Identification and Characterization of Fungi

All the fungal colonies observed were identified using light microscopy ( $\times 40, \times 100$ ) and by staining with cotton blue reagent (Tripathi, 2006). The hyphae, mycelia and conidial structures were studied to aid in identification of the fungi. DNA extraction, PCR Amplification and sequencing were used to identify the fungi as described by Pant and Adholeya (2007).

### Sub Culturing to Obtain Pure Cultures of Fungi

All the fungal colonies were sub cultured on fresh potato dextrose agar medium using streak and pour plate method. Incubation was done at 30°C for 7 days in order to obtain pure cultures.

### Molecular Identification of Fungal Strains Nucleic Acid (DNA) Extraction, Concentration and Purity

Following the instructions of the manufacturer (Epicenter), the Genomic DNA of the fungi was extracted. The Epicenter kit was used to extract the Genomic DNA. About 1-5 mg each of fresh fungal mycelia were either processed immediately or frozen at -70°C and diluted with 1 µl of mixture of 50 µg/µl Proteinase K and 300 µl of Tissue and Cell Lysis Solution and homogenized before transferring to a microcentrifuge tube. This was mixed thoroughly before incubating the Eppendorf tubes at 65°C for 15 minutes. The tubes were vortexed every 5 minutes. The samples were placed on ice for 3-5 minutes. About 175 µl of MPC Protein Precipitation Reagent was added to 300 µl of the lysed sample and vortexed vigorously for 10 seconds. The debris was pelleted by centrifugation at 4°C for 10 minutes at 10,000 x g in a microcentrifuge. The supernatant was transferred to a clean microcentrifuge tube and the pellet discarded. About 500 µl of isopropanol was added to the recovered supernatant. The tubes were inverted 30-40 times. The total nucleic acids were pelleted by centrifugation at 4°C for 10 minutes in a microcentrifuge. The isopropanol was carefully poured off without dislodging the total nucleic acid pellet and rinsed twice with 70% ethanol, being careful to not dislodge the total nucleic acid pellet. The residual ethanol was removed with a pipette. The total nucleic acids were re-suspended in 35 µl of TE Buffer. Genomic DNA concentration was measured using Biophotometer (Eppendorf, Germany). This was achieved by taking the sample's optical density (OD) at a wavelength of 260 nm. One OD was equivalent to 50 µg / mL of DNA. On the other hand, the sample's purity was determined by taking the ratio of 260 nm and 280 nm optical densities. Samples with ratio between 1.8 nm and 2.0 nm were considered pure (White *et al.*, 1990).

### PCR Amplification and Sequencing

Two universal oligonucleotide primers were used for polymerase chain amplification. They were the ITS-1 forward primer (5' TCC GTA GGT GAA CCT GCG G3') and the ITS-4 reverse primer (5' GCT GCG TTC TTC TTG ATC GAT GC 3'). The primers were developed based on descriptions by White *et al.*, (1990) and were supplied by 1<sup>st</sup> Base Laboratories, Malaysia. Amplification reactions were conducted in 50µl reaction mixture containing a final concentration 1 µl of 10 X PCR buffer (NovaTaq), 4µl of MgCl<sub>2</sub>, (25mM) 0.5µl of Nova *Taq* polymerase (100U), 1µl of dNTP (10Mm), 39.5µl of distilled deionised waer and 4µl of genomic DNA. All the reagents above were obtained from Novagen Lab, USA (Saitoh *et al.*, 2006).

Polymerase Chain Reaction (PCR) amplifications were performed using the BIOMETRA Tpersonal/Tprofessional Thermocycler (Germany) with

an initial denaturation of 5 minutes at 95°C followed by 30 cycles of denaturation at 95°C for 45 seconds, annealing at 55°C for 90 secs and extension at 72°C for 90 secs, with a final extension step at 72°C for 7 minutes. After amplification, the products were used immediately or stored at 4°C before sequencing (Saitoh *et al.*, 2006).

### Electrophoresis

Aliquots of 5 µl of each PCR product were subjected to electrophoresis on a 1.8% horizontal agarose gel (Promega) in a 1 X TBE buffer at 70V for 90Mins, depending on the size of the amplified fragment from the primer. The gel was stained in 1µl of Gel RED for 15 to 20 minutes. A 1kb marker ladder (Fermentas) was used as molecular weight standard to estimate the size of the ITS 1 regions amplified. The gel was photographed using an Ultraviolet transilluminator and the ITS products were photographed using the UVDI Analyser (Major Science, TAIWAN).

### Molecular Sequence Analysis

The PCR products were commercially purified and sequenced by 1<sup>st</sup> Base Laboratories (Malaysia). The resultant nucleotide sequences were assembled and manually edited with BioEdit software, version 7.0.9. Furthermore, the nucleotide sequences were subjected to sequence homology search using BLAST tool (Saitoh *et al.*, 2006).

In performing the search, sequence with lower *E*-value score of <10<sup>-3</sup> and ≥ 70% were considered homologous. Nucleotide sequences generated in this study were also deposited in NCBI- GenBank database and were assigned accession numbers.

### Pretreatment

Pretreatment was carried out (Shi *et al.*, 2008) to delignify the feedstocks and dissolve the hemicelluloses while conserving the cellulose content.

### Submerged Cultivation (SMC)

From the results of the screening of the native fungi for lignocellulolytic activity, and the determination of the effect of temperature on the growth condition and cellulase activity, two fungi, namely, *T.aureoviride* UPM 09 (JN811063) (UPMC 389) and *F. equiseti* strain UPM-09 (JN811061) were selected and used for the pretreatment in this study. The strains were pre-cultured on potato-dextrose agar (PDA) plates at 30 °C for 7 days. Seven grams each of the feedstocks, namely, rice husk, rubber Wood and Oil Palm Empty Fruit Bunch were weighed. The minimal salts medium (100 ml) was prepared and dissolved in one liter of distilled water (g/l): Urea (0.3), KH<sub>2</sub>PO<sub>4</sub> (2.0), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (1.4), MgSO<sub>4</sub>.7H<sub>2</sub>O (0.3), Peptone (0.75), Yeast extract (0.25), CaCl<sub>2</sub>.2H<sub>2</sub>O (0.002). Trace elements: FeSO<sub>4</sub>.7H<sub>2</sub>O (0.005), MnSO<sub>4</sub>.7H<sub>2</sub>O (0.0016), ZnSO<sub>4</sub>.7H<sub>2</sub>O.0014), CoCl<sub>2</sub> (0.002) were added. The substrates were added into each bottle. pH was recorded and the bottles were sterilized at 121°C for 15 minutes in an autoclave. The substrates were inoculated with fungi (spores/ml). Spore suspensions were prepared by washing the agar surface with 10 ml of sterile distilled water.

Spore counts were determined with hemacytometer (Hausser Scientific, Horsham, PA) and the final spore inocula had a concentration of  $5.6 \times 10^9$  for spores/ml for *T. aureoviride* UPM 09 (JN811063) (UPMC 389) and  $9.6 \times 10^9$  spores/ml *F. equiseti* strain UPM-09 (JN811061) for both the individual and mixed culture. The bottles were incubated at 30°C at 150 RPM for 15 days. The control samples were not inoculated with any fungal spores after autoclaving. About 2.5 milliliters of the supernatant were withdrawn daily and centrifuged at 10,000 rpm for 5 min for enzyme and glucose analysis.

### Solid State Cultivation (SSC)

Pretreatments were carried out in 250 mL Erlenmeyer flasks, each of which contained the feedstocks, namely, rice husk (RH), rubber Wood (RW) and Oil Palm Empty Fruit Bunch (EFB) (7 g) with 0.6-1.00 mm particle size. Distilled water (12 mL) was added to obtain the appropriate substrate moisture content (75 %). The flasks were sterilized at 121 °C for 20 min then cooled and aseptically before inoculating with spores inoculums of individual and mixed culture. Para film was wrapped around flasks to act as a barrier against moisture loss and contamination. Small perforations were made to the film to avoid moisture condensation and allow ventilation of chambers. Flasks were maintained statically at 30°C for 15 days. A set of non-pretreated sterilized substrates for each sample were used as control. About 2.5 milliliters of the supernatants were withdrawn daily and centrifuged at 10,000 rpm for 5 minutes for enzyme and glucose analysis at least in triplicate. After pretreatment, the flasks were stored at 4°C before composition analysis and enzymatic hydrolysis.

Chemical compositions (%) of the feedstocks, rice husk (RH), rubber Wood (RW) and Oil Palm Empty Fruit Bunch (EFB) were determined before and after pretreatment relative to lignin, cellulose, and hemicellulose content.

### Enzyme Hydrolysis

The untreated and pretreated samples with *Trichoderma aureoviridae* strain UPM 09 (JN811063) (UPMC 389) and *Fusarium equiseti* strain UPM 09 (JN811061) and a pretreatment combination of both fungi were used for enzymatic hydrolysis.

Enzymatic hydrolysis was performed following NREL laboratory analytical procedure LAP-008 (Dowe and McMillan, 2001). Enzymatic hydrolysis experiments were carried out using commercial cellulase (Celluclast 1.5 L, produced by *T. reesei*) with activity of 70 FPU / mL, supplemented with β-glucosidase (Novozyme 188, produced by *Aspergillus niger*) with activity of 122 CBU/ mL. The Filter Paper Unit (FPU) was used to define the enzyme activity that will produce reducing sugar equivalent to 2 mg of glucose (Adney and Baker, 1996) and 1 CBU was defined as the amount of enzyme that forms 2 μmole of glucose per min from cellobiose.

Enzymatic hydrolysis experiments were carried out in 250 mL bottles. Each bottle was loaded with 1 % w/w effective cellulose content, 1 % w/v yeast extract, 2 % w/v peptone and 0.05 M citrate buffer (pH 4.8) in

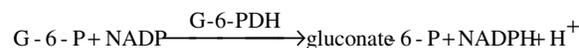
a final working weight of 50 grams and autoclaved at 121 °C for 15 min. After cooling at room temperature, cellulase enzyme was added to each of the bottles at a dose of 25 FPU / g cellulose; and was supplemented with β-glucosidase with a dose of 60 CBU / g cellulase at a ratio of 1:2 v/v to avoid inhibition due to cellobiose accumulation. The reaction mixtures were incubated in a rotary shaker set at 150 rpm and 50 °C for 168 hours. About 2.5 milliliters of the samples were taken after 24, 48, 72, 96, 120, 144 and 168 h and stored in capped tubes at -20 °C until needed. The capped tubes were boiled in a water bath for exactly 5 min to inactivate cellulase and chilled on ice. Hydrolyzed samples were centrifuged at 10,000 rpm for 5 min.

### Determination of Glucose

The glucose produced during pretreatment and enzymatic hydrolysis was measured by hexose kinase method using Randox analytical kits on an automated glucose analyzer (Selectra XL, Neitherrands). The enzymes kit contains two enzymes, Hexose kinase (HK) and glucose-6-phosphate dehydrogenase (G-6-PDH) together with buffer, adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADP). The glucose analysis was based on the following principle (Sun and Cheng, 2002):



Hexose kinase catalyses the phosphorylation of glucose to glucose-6-phosphate



Glucose-6-phosphate dehydrogenase oxidizes glucose-6-phosphate in the presence of NADP to gluconate-6-phosphate and NADP is reduced to NADPH. This reaction is specific to glucose, thus no other carbohydrate in the solution is oxidized. The rate of NADPH formation is directly proportional to the glucose concentration and can be measured photometrically. The glucose yield was calculated by the equation: 18mg/dL = 1 m mol/ L as described by the manufacturer.

### Determination of Protein

The protein content was determined using Biorad assay with bovine serum albumin as standard.

### Determination of Reducing Sugar by Dinitrosalicylic Acid (DNS) Method

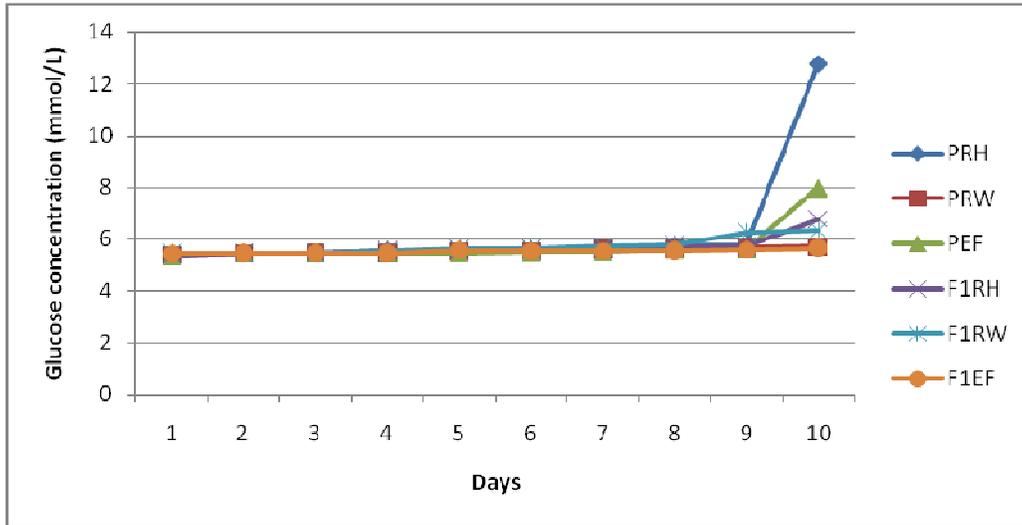
Total reducing sugar was determined by the 3,5-dinitrosalicylic acid (DNS) method using glucose as the standard (Ghose, 1987). About 1 mL of sample and 3 mL DNS were mixed, boiled for exactly 5 min, cooled and diluted in water. Color formation was determined by measuring absorbance against the water blank at 540 nm. Glucose standard made up and diluted like samples to varying concentrations. Reducing sugar yield was calculated using the following equation:

$$\text{Reducing sugar yield (\%)} = \frac{\text{amount of glucose} \times 0.9 \times 100}{\text{H}}$$

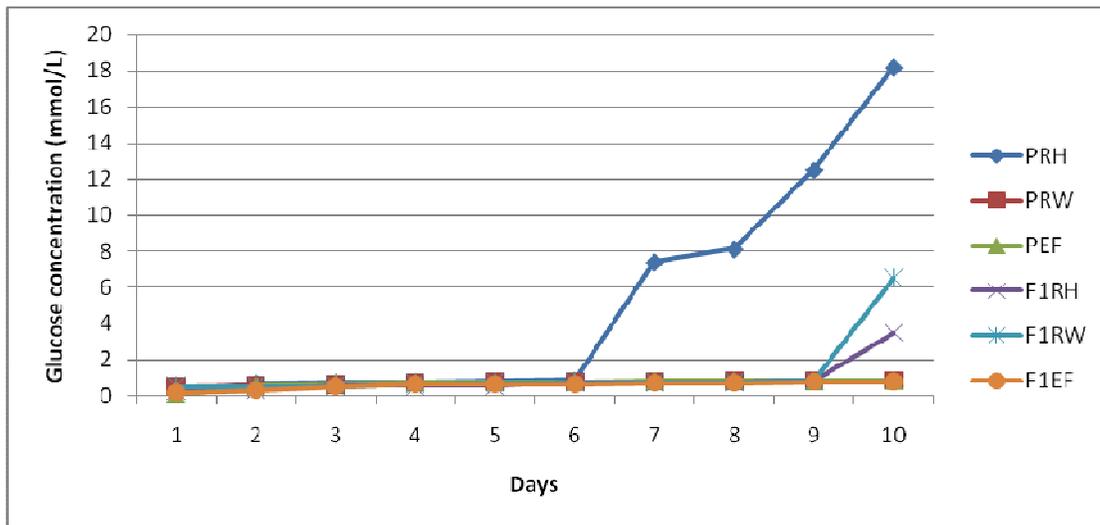
Where: H = Cellulose and hemicelluloses

**RESULTS AND DISCUSSION**

The amount of glucose produced during pretreatment of the feedstocks is presented in Figures 1 and 2. While the corresponding amount of protein produced from the feedstocks is presented in Figures 3 and 4 by SSC and SMC using *F. equiseti* UPM 09 (F1) and *T. aureoviride* (P) UPM 09. The reason for lower glucose production in SSC (Figure 2), however, may be due to consumption of glucose by the fungi because of proximity of the fungi to carbon source in this case the feedstocks.



**Figure 1: Amount of glucose produced from rice husk (RH), rubber wood (RW) and oil palm empty fruit bunch (EFB) pretreated with F1 (*F. equiseti*) and P (*T. aureoviride*) by submerged cultivation (SMC).**



**Figure 2: Amount of glucose produced from RH, RW and EFB pretreated with F1 and P by solid state cultivation (SSC).**

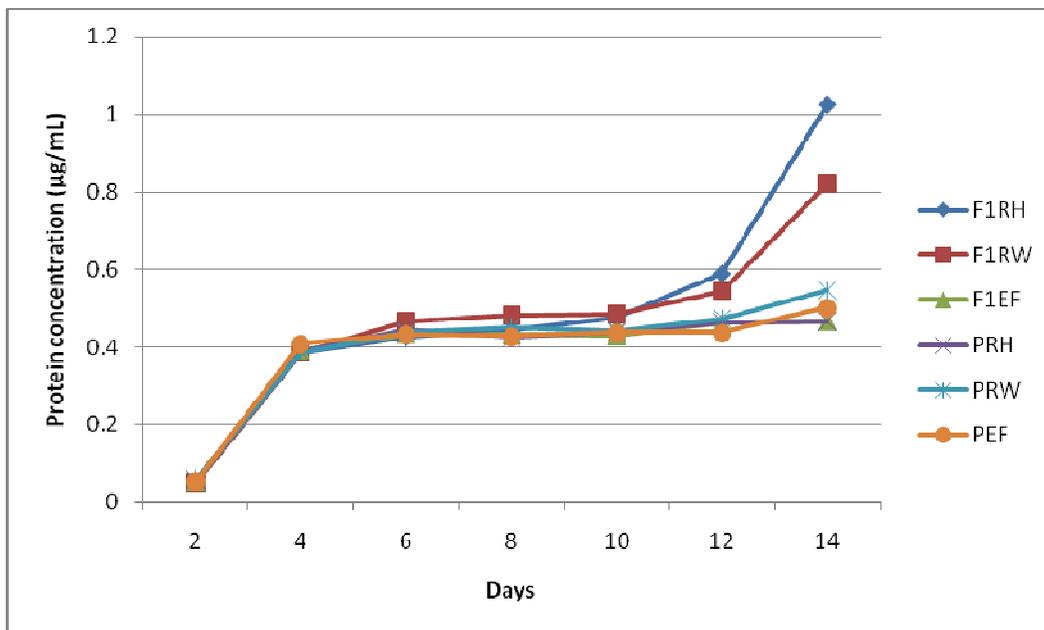


Figure 3: Protein produced from pretreatment of RH, RW and EFB by F1 and P using SMC.

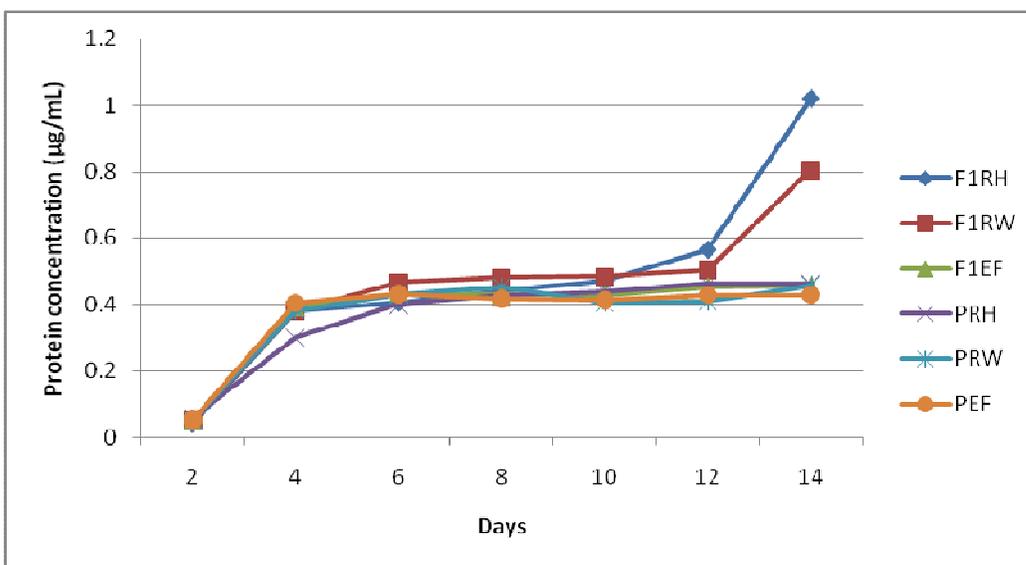


Figure 4: Protein produced from pretreatment of RH, RW and EFB by F1 and P using SSC.

This result signifies that the two fungal strains can perform pretreatment and hydrolysis simultaneously, especially *T. aureoviride* UPM 09 that produced high amount of glucose from RH in both SSC and SMC thus making this finding in line with a number of studies that revealed high levels of proteins together and cellulases like *Aspergillus* species thereby making it an ideal organism for industrial applications as reported by Sun and Cheng (2002). However, the overall production of protein by *T. aureoviride* UPM 09 (P) and *F. equiseti* UPM (F1), respectively, using RH and RW was high in both SMC and SSC.

In order to evaluate the effect of pre-treatment with *T. aureoviride* strain UPM 09 and *F. equiseti* strain UPM 09 using the best samples from the individual and consortium of the on enzymatic hydrolysis of

pretreated RH, RW and EFB, the fermentable sugar yields were determined after enzymatic hydrolysis for 0, 3, 24, 48, 72, 96, 120, 144, and 168 hours. Zhang *et al.* (2007) reported that the root causes of the recalcitrance of lignocellulose to enzymatic hydrolysis could be mainly attributed to: (1) low accessibility of (micro-) crystalline cellulose fibers, which restricts cellulase activity, and (2) the presence of lignin (mainly) and hemicellulose on the surface of cellulose, which prevents cellulase from accessing the substrate; based on a their studies amorphous cellulose has a high reactivity because its enzymatic hydrolysis pattern is completely different from that of crystalline cellulose and the synergy among exoglucanase and endoglucanase is unimportant to amorphous cellulose hydrolysis.

The results of this study show that the enzymatic hydrolysis yield of RH and RW is considerably affected by the cultivation time and the reducing sugar yield heavily depends on the extent of delignification and hemicellulose removal from the lignocellulosic materials due to removal the physical protective coat of cellulose and consequently improved cellulose digestibility. This explains why sample pretreated by consortium of *T.aureoviride* strain UPM 09 and *F. equiseti* strain UPM 09 by SSF resulted in the highest sugar yield (98%). It was reported that after 120 days of cultivation by a newly isolated fungus

*Echinodontium taxodii* 2538 on two native woods: *Chinese willow* (hard wood) and *China-fair* (soft wood), the enzymatic hydrolysis yield showed significant increase (4.7-fold for hard wood and .3-fold for soft wood) (Teramoto *et al.*, 2008). Lee *et al.* (2007) also reported lower sugar yields (21.01 %, 14.91 %, and 15.03 %) from soft wood *Pinus densiflora* pretreated with *Stereum hirsutum*, *Polyporus brumalis*, and *Ceriporia lacerate*, respectively compared to RW and RH pretreated with *T.aureoviride* strain UPM 09 and *F. equiseti* strain UPM 09 individually (20 -22%.) as in Table 1.

**Table 1: Reducing Sugar (mg) concentrations of pretreated Samples after enzymatic hydrolysis**

TIME (Hours)	F1PRH (SSF)	F1RW (SMF)	PRW (SMF)	F1PRH (SMF)	PRW (SSF)	Untreated (RW)	CMC (Positive Control)
168	29.4 (98%)	13.2 (42.5%)	27.0 (90%)	11.25 (37.5%)	24.6 (83%)	3.75 (11.25%)	30.1 (99.8%)

**CONCLUSION**

The result of this study, therefore, showed that *Trichoderma aureoviride* strain UPM 09 (JN811063) and *Fusarium equiseti* strain UPM 09 (JN811061) possess potentials for the pretreatment of

lignocellulosic biomass and can also be used for enzymatic hydrolysis of pretreated biomass as potential substrates for production of biofuels. . The authors declare that there is no conflict of interest.

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