

Effect of Different Microbial Strains on Biological Pretreatment of Sugarcane Bagasse for Enzymatic Hydrolysis

Achiraya Jiraprasertwong, Erdogan Gulari, Sumaeth Chavadej

Abstract—Among agricultural residues, sugarcane bagasse is one of the most convincing raw materials for the production of bioethanol due to its availability, and low cost through enzymatic hydrolysis and yeast fermentation. A pretreatment step is needed to enhance the enzymatic step. In this study, sugarcane bagasse (SCB), one of the most abundant agricultural residues in Thailand, was pretreated biologically with various microorganisms of white-rot fungus—*Phanerochaete sordid* (SK 7), *Cellulomonas* sp. (TISTR 784), and strain A 002 (*Bacillus subtilis* isolated from Thai higher termites). All samples with various microbial pretreatments were further hydrolyzed enzymatically by a commercial enzyme obtained from *Aspergillus niger*. The results showed that the pretreatment with the white-rot fungus gave the highest glucose concentration around two-fold higher when compared with the others.

Keywords—Sugarcane bagasse, Microorganisms, Pretreatment, Enzymatic hydrolysis.

I. INTRODUCTION

AS the depletion of energy resources, global climate change, and dramatic increases in fuel prices, biomass conversion technologies can help in solving these severe problems. Among biomass materials, lignocellulosic biomass is a suitable material for the production of biofuels (bioethanol, biobutanol, and biogas) according to its availability in a large quantity and without disturbance in food supply; furthermore, it generates very low net greenhouse emissions [1].

Lignocellulosic biomass mainly consists of cellulose, hemicelluloses, and lignin. The process of lignocellulosic biomass conversion requires: (1) a pretreatment step to breakdown the lignin and the crystalline structure of cellulose; (2) a hydrolysis step of carbohydrate polymers to produce free sugars; and (3) a microbial fermentation step of produced sugars [2], [3]. Regarding the complex structure of lignocellulosic materials, most studies have focused on the pretreatment step.

Various pretreatment methods are both chemical pretreatment and physical pretreatment methods requires expensive equipment, high operational cost and has negative

impacts on environments [4], [5]. Accordingly, biological pretreatment is a promising method because this technique can be operated under mild conditions, and requires low energy consumption as well as environmental friendly [2], [6], [7].

In this study, sugarcane bagasse (SCB) was biologically pretreated with various stains including the white-rot fungus—*Phanerochaete sordid* (SK 7), the *Cellulomonas* sp. (TISTR 784), and A 002 (*Bacillus subtilis* isolated from Thai higher termites). Subsequently, the pretreated bagasse (PSCB) samples were further hydrolyzed by a commercial enzyme obtained from *Aspergillus niger* to produce glucose production.

II. PROCEDURE

A. Materials and Microorganism

A sugarcane bagasse sample was obtained from Saraburi Sugar Co., Ltd, Thailand and dried in an oven. The dried SCB sample was milled and screened into 40–60 mesh sizes (0.40–0.25 mm). The milled bagasses were dried and stored under dry condition in plastic boxes until use.

All chemicals used were in reagent grade. The *Bacillus subtilis* (A 002), was isolated from Thai Higher Termites, *Microceroterme* sp. [8]. The culture medium was a 65 modified DSMZ broth medium containing (L^{-1}) 5 g of carboxymethyl cellulose (CMC), 4 g of yeast extract, and 10 g of malt extract with an initial pH of 7.2 [9].

Cellulomonas sp. (TISTR 784) from Thailand Institute of Scientific and Technological Research (TISTR) was cultivated in a modified NCBE broth medium [10] consisting of (L^{-1}) 5 g of CMC, 1 g of $NaNO_3$, 1 g of K_2HPO_4 , 1 g of KCl, 0.5 g of $MgSO_4$, and 0.5 g of yeast extract with an initial pH of 7.2.

The fungus, SK 7, was isolated from *Phanerochaete sordid* of white-rot fungus [11]. It was cultured on a modified medium containing 2% (w/v) malt extract agar (MEA) [12] and 20 pieces cut from actively growing mycelium were used to inoculate in 200 mL of a malt extract broth (MEB) in a 250 mL Erlenmeyer flask at 30°C for 10 d. The mixer was homogenized (Omnimixer, Thailand) at 30,000 rpm for 20 s in triplicate.

B. Biological Pretreatment Experiments

A single loop of colonies was grown into a 250 mL Erlenmeyer flask containing 50 mL of the 65 modified DSMZ broth medium at pH 7.2 for strain A 002, and contained 50 mL of the modified NCBE broth medium, pH 7.2 for TISTR 784. After a static cultivation at 37°C for 12 h, 50 mL of any

A. J. is with the Petroleum and Petrochemical College, and the Center of Excellence on Petrochemical and Materials Technology, Bangkok 10330 Thailand (e-mail: achiraya.jp@gmail.com).

E. G. is with University of Michigan, Ann Arbor, MI 48105 USA (e-mail: gulari@umich.edu).

S. C. is with the Petroleum and Petrochemical College, and the Center of Excellence on Petrochemical and Materials Technology, Bangkok 10330 Thailand (phone: +66(0)221-84139; e-mail: sumaeth.c@chula.ac.th).

prepared inoculum was transferred into a 500 mL bottle with a screw cap containing 450 mL of the production medium (65 modified DSMZ broth medium and modified NCBE broth medium, pH 7.2) and the 40-mesh sugarcane bagasse sample was added to obtain 5% (w/v). The mixture was incubated at 37°C for 48 h in a shaking incubator with an agitation rate of 180 rpm. Finally, the mixture was filtered, washed, and dried in an oven at 105°C overnight.

In fungal pretreatment, the experiment was carried out in a 250 mL Erlenmeyer flask containing 5% (w/v) of bagasse (SCB) and 0.025 mL of corn steep liquor. The humidity was adjusted to 100% and the mixer was then autoclaved at 121°C and 15 psi for 15 min. Culture was maintained at 30°C for 20 d, then washed with 200 mL of DI water and dried at 105°C overnight. In addition, a set of unpretreated sterilized bagasses were used as control [11].

C. Enzymatic Hydrolysis Experiments

The pretreated sugarcane bagasse samples by different strains were further hydrolyzed by the commercial enzyme from *Aspergillus niger* (Sigma Chemical Co.) with 25 U/g dry substrates in an acetate buffer solution at pH 4.8 with raw materials concentration of 1% (w/v) and 0.01% sodium azide was added to prevent the contamination of microorganisms [13]. The experiment was carried out at 37°C for 72 h with an agitation rate of 180 rpm.

D. Analysis of Glucose Concentration

At any desired time interval of the enzymatic hydrolysis, the reaction solution was withdrawn to analyze for glucose concentration. The samples were filtered through a 0.22 µm filter paper and the filtrate samples were taken for the analysis of glucose concentration by using a HPLC with a BIORAD HPX-87H column with a 0.005M H₂SO₄ solution as a mobile phase at a constant flowrate of 0.6 mL/min, and the system equipped with a refractive index detector (Model 6040 XR, Spectra-Physics, USA) at a temperature at 65°C.

E. Surface Morphology Examination

A field emission scanning electron microscope (FE-SEM, Hitachi, S-4800 model) was used to examine the surface morphology of both untreated and pretreated sugarcane bagasses. The samples were coated with Pt and the FE-SEM was operated at a voltage of 2 kV.

F. Fourier Transform Infrared Spectrometer Analysis

The dried bagasse samples were mixed with KBr, and made in the form of pellets using a pressure of 7000 ton/in². A FTIR (Nicolet Nexus 670) was operated at a spectral resolution of 4 cm⁻¹ and 64 scans were taken per sample.

G. XRD Analysis

X-ray diffraction (XRD) was used to measure the crystallinity of all sugarcane bagasse samples. The samples were scanned and recorded by using a Rigaku X-ray diffractometer system (RINT-2200). All samples were scanned from 2θ = 5° to 50°, with a scan step of 0.02 (2θ), at

40 kV and 30 mA. The crystalline index (CrI) is defined as follows:

$$\text{CrI}(\%) = \frac{I_{002} - I_{\text{am}}}{I_{002}} \times 100$$

where I₀₀₂ is the crystalline peak of the maximum intensity at 2θ between 22° and 23° and I_{am} is the minimum intensity at 2θ between 18° and 19° [5].

III. RESULTS AND DISCUSSION

A. FTIR Results

The FTIR spectra of various bagasse samples are shown in Fig. 1. The broad band in the range of 3600–3100 cm⁻¹ is defined as a strong hydrogen bonding (O–H) stretching in cellulose [14]–[16]. In addition, the peak spectrum around 1800–900 cm⁻¹ is assigned to fingerprint the regions of various functional groups of cellulose, hemicellulose and lignin. The FTIR spectra after the pretreatment with different strains (Fig. 2) showed significantly changed; especially the peak at 1738 cm⁻¹ (unconjugated C=O in xylans or hemicelluloses) of the SK 7 treated decreased in the intensity after the pretreatment. Moreover, the intensities of the peaks at 1505 (aromatic skeletal in lignin), 1462 (C–H deformation in lignin), 1330 (C–H vibration in cellulose and C–O vibration in syringyl derivatives), 1244 (syringyl ring and C–O stretch in lignin and C–O linkage in guaiacyl aromatic methoxyl groups which is the main constituting units of lignin), and 1122 (aromatic skeletal and C–O stretch) cm⁻¹ decreased after the pretreatment step which clearly observed at bands 1505, 1330, and 1244 cm⁻¹, indicating lignin degradation. For the C–H deformation in cellulose (at 898 cm⁻¹), the FTIR spectra after pretreatment were not observed obviously due to cellulose was not changed significantly after biological pretreatment.

B. XRD Results

Table I shows the change of crystallinity of SCB after biological pretreatment and enzymatic steps. Generally, all components of cellulose, hemicellulose and lignin consist of both crystalline and amorphous forms [17], [18]; however, the X-ray measurement of CrI is still the best way to determine the crystallinity of the entire materials [5]. After the microbial pretreatment, the increase in CrI of SCB after the pretreatment with A 002 can conclude that the cellulose might become more exposed after pretreatment [19] because the degradation and modification of the amorphous cellulose [5], [20]. Nevertheless, which the surface became smoother than before,

TABLE I
THE CRYSTALLINITY INDEX OF SUGARCANE BAGASSES

Pretreatment Method	CrI, %		
	Untreated Sugar Bagasse	After Biological Pretreatment	After Enzymatic Hydrolysis
A 002	69.12	70.24	66.44
TISTR 784	69.12	63.95	70.05
SK-7	69.12	64.18	67.34

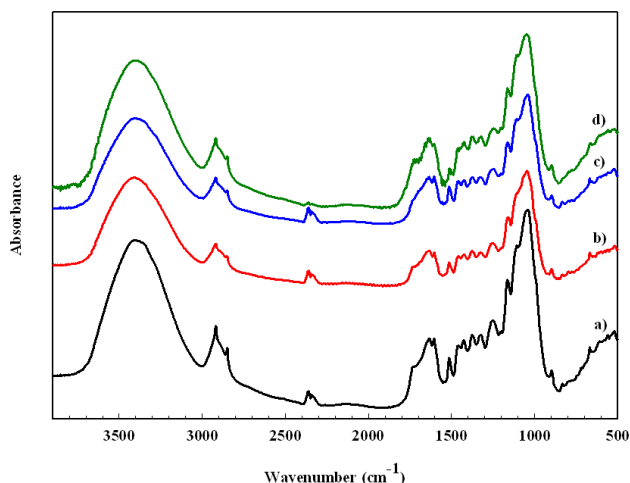


Fig. 1 FTIR spectra of sugarcane bagasses a) Untreated bagasse; b) A 002 treated bagasse; c) TISTR 784 treated bagasse; and d) SK-7 treated bagasse

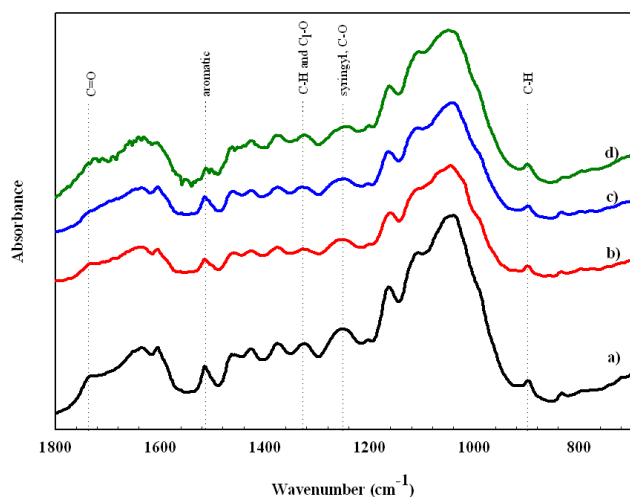


Fig. 2 FTIR spectra of biological pretreatment sugarcane bagasses in the range of 700–1800 cm^{-1} a) Untreated bagasse; b) A 002 treated bagasse; c) TISTR 784 treated bagasse; and d) SK-7 treated bagasse

the depletion of CrI values after the pretreatment with TISTR 784 and SK 7 can be explained by a reduction in the intra- and intermolecular hydrogen bonds [16] to change the cellulose structure from crystalline to amorphous form, resulting in better accessibility for the hydrolysis process. After the enzymatic hydrolysis process, the CrI values increased with the pretreatment using SK 7 and TISTR 784 while the CrI value adversely decreased with the pretreatment using A 002. The reason the increase in the CrI might be probably that the amorphous part was further hydrolyzed to liberate glucose.

C. Morphological Structure Change

Fig. 3 shows the significant difference in surface morphology of SCB after the biological pretreatment with different strains. The surface structure of the sample after the pretreatment with A 002 was quite similar to that of the TISTR 784 pretreatment (Fig. 3 (c)) but the surface of the

PSCB with SK 7 was much rougher indicating that the fungal pretreatment can degrade SCB much more efficiently by altering the chemical and physical structures of lignin to leave the wood with the white fibrous appearance of fungal mycelia [21]. As showed in Fig. 3, the SK 7 pretreatment increase the surface area of PSCB, leading to an increase in accessibility to enzyme during the enzymatic hydrolysis step.

In Table II, variable data from different microbial pretreatment are shown. The highest total reducing sugar was obtained at $0.65 \text{ g}\cdot\text{L}^{-1}$ from SK-7 pretreatment which was found to be around two-fold and three-fold more than untreated sugarcane bagasse and the other the biological pretreatments, respectively. In general, biological pretreatment is not an efficient method for pretreatment because some microbes also consume cellulose for growth leading to loss of sugar recovery [23]. From the results, it can conclude that both strain A 002 and TISTR 784 are not appropriate for pretreatment of sugarcane bagasse while pretreatment with SK-7 significantly affect on glucose production; furthermore, the total sugar in this study when compared with various biological pretreatment (Table III) was quite high which implied that pretreatment with SK-7 was a promising method which enhance efficient hydrolysis step for sugarcane bagasses.

TABLE II
SUGARCANE BAGASSES IN BIOLOGICAL PRETREATMENT

Treatments	Cellulose (%)	Lignin (%)	Glucose ($\text{g}\cdot\text{L}^{-1}$)	Total sugar ($\text{g}\cdot\text{L}^{-1}$)
Untreated	46.9 ± 5.7	14.5 ± 3.2	0.27	0.34
A 002	46.3 ± 0.5	15.1 ± 0.3	0.25	0.25
TISTR 784	43.3 ± 3.4	17.4 ± 3.1	0.26	0.26
SK-7	42.6 ± 0.6	10.6 ± 1.1	0.53	0.65

TABLE III
COMPARISON OF THE TOTAL REDUCING SUGAR UNDER VARIOUS BIOLOGICAL PRETREATMENT

Substrates	Treatment	Total sugar ($\text{mg}\cdot\text{g biomass}^{-1}$)	Reference
Sugarcane bagasse	<i>Phanerochaete sordid</i> , SK-7	65	Present work
Sugarcane trash	<i>Cellulomonas cartae</i>	8.79	[24]
Sugarcane trash	<i>Cellulomonas uda</i>	7.15	[24]
Sugarcane bagasse	<i>Corioliopsis caperata</i> RCK2011	192.52	[25]

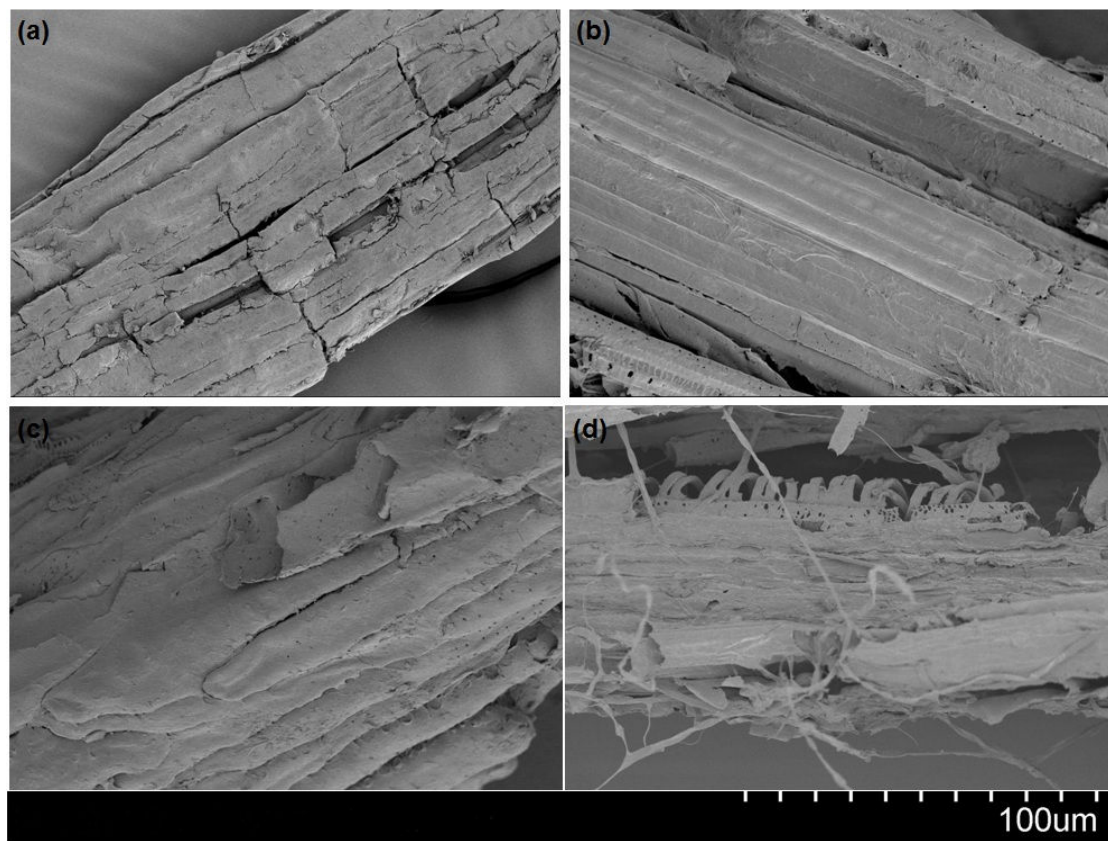


Fig. 3 SEM micrographs of untreated and pretreated sugarcane bagasse using magnification of 400 (a) untreated bagasse; (b) A 002 treated; (c) TISTR 784 treated; and (d) SK-7 treated

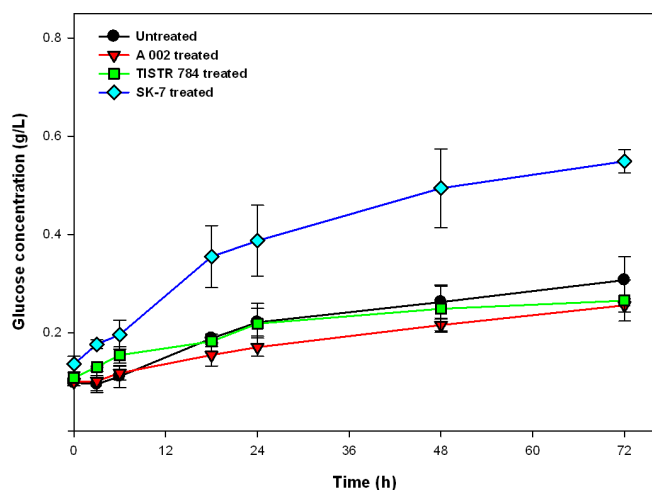


Fig. 4 Glucose concentration during enzymatic hydrolysis of unpretreated and pretreated with various strains in sugarcane bagasses

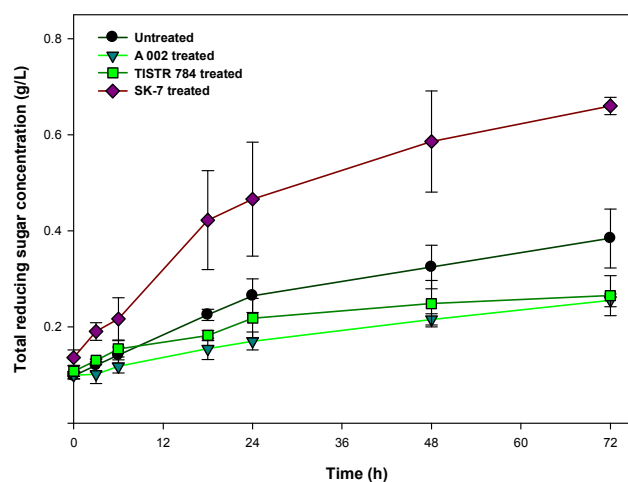


Fig. 5 Total reducing sugar concentration during enzymatic hydrolysis of unpretreated and pretreated with various strains in sugarcane bagasses

IV. CONCLUSIONS

Pretreatment methods can improve an efficiency of hydrolysis on lignocelluloses, resulting in increasing digestion and accessibility in hydrolysis process [26]. Although many literatures report that biological pretreatment is a slow process and obtains lower products than other methods [5], [27], [28], this process is a promising method. The results from this study

indicate that fungal is the best candidate for biological pretreatment by reason of gives the highest glucose concentration as well as total sugars. Furthermore, strain A 002 is not appropriate for biopretreatment because it consumes cellulose for their growth while continually produce cellulase enzyme.

ACKNOWLEDGMENT

This work was supported by Thai Oil Pub Co., Ltd.; the Center of Excellence on Petrochemicals and Materials Technology, Chulalongkorn University and Chula Unisearch, Chulalongkorn University. The authors would like to thank Saraburi Sugar Co., Ltd. for providing the materials. The authors also thank to the Petroleum and Petrochemical College, Chulalongkorn University for offering the laboratory during this work. Moreover, the authors gratefully acknowledged the Thailand Institute of Scientific and Technological Research (TISTR) and the Department of Botany, Faculty of Science Chulalongkorn University for offering the microbes.

REFERENCES

- [1] P. Kumar, D. M. Barrett, M. J. Delwiche, and P. Stroeve, "Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production," *Industrial & Engineering Chemistry Research*, vol. 48, no. 8, pp. 3713-3729, 2009.
- [2] Y. Sun, and J. Cheng, "Hydrolysis of lignocellulosic materials for ethanol production: a review," *Bioresource technology*, vol. 83, no. 1, pp. 1-11, 2002.
- [3] N. Narayanaswamy, P. Dheeran, S. Verma, and S. Kumar, "Biological Pretreatment of Lignocellulosic Biomass for Enzymatic Saccharification," *Pretreatment Techniques for Biofuels and Biorefineries*, pp. 3-34: Springer, 2013.
- [4] J. Lee, K. Gwak, J. Park, M. Park, D. Choi, M. Kwon, and I. Choi, "Biological pretreatment of softwood *Pinus densiflora* by three white rot fungi," *Journal of Microbiology-Seoul-*, vol. 45, no. 6, pp. 485, 2007.
- [5] C. Xu, F. Ma, X. Zhang, and S. Chen, "Biological pretreatment of corn stover by *Ipex lacteus* for enzymatic hydrolysis," *Journal of agricultural and food chemistry*, vol. 58, no. 20, pp. 10893-10898, 2010.
- [6] L. P. Ramos, "The chemistry involved in the steam treatment of lignocellulosic materials," *Química Nova*, vol. 26, no. 6, pp. 863-871, 2003.
- [7] P. Alvira, E. Tomás-Pejó, M. Ballesteros, and M. Negro, "Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review," *Bioresource technology*, vol. 101, no. 13, pp. 4851-4861, 2010.
- [8] K. Taechapoempol, T. Sreethawong, P. Rangsunvigit, W. Namprohm, B. Thamprajamchit, S. Rengpipat, and S. Chavadej, "Cellulase-producing bacteria from Thai higher termites, *Microcerotermes* sp.: enzymatic activities and ionic liquid tolerance," *Applied biochemistry and biotechnology*, vol. 164, no. 2, pp. 204-219, 2011.
- [9] M. Wenzel, I. Schöning, M. Berchtold, P. Kämpfer, and H. König, "Aerobic and facultatively anaerobic cellulolytic bacteria from the gut of the termite *Zootermopsis angusticollis*," *Journal of applied microbiology*, vol. 92, no. 1, pp. 32-40, 2002.
- [10] K. Apun, "Cellulase production," 1995, Available online at <http://www.ncbe.reading.ac.uk/ncbe/protocols/PRACBIOTECH/PDF/cellprod.pdf>.
- [11] V. Sripat, "pretreatment of *Eucalyptus* wood using tropical strains of white rot fungi for kraft pulping," Chulalongkorn University, Thailand, 2013.
- [12] C. Liew, A. Husaini, H. Hussain, S. Muid, K. Liew, and H. Roslan, "Lignin biodegradation and ligninolytic enzyme studies during biopulping of *Acacia mangium* wood chips by tropical white rot fungi," *World Journal of Microbiology and Biotechnology*, vol. 27, no. 6, pp. 1457-1468, 2011.
- [13] K.-L. Chang, J. Thitikorn-amorn, J.-F. Hsieh, B.-M. Ou, S.-H. Chen, K. Ratanakhanokchai, P.-J. Huang, and S.-T. Chen, "Enhanced enzymatic conversion with freeze pretreatment of rice straw," *Biomass and Bioenergy*, vol. 35, no. 1, pp. 90-95, 2011.
- [14] K. Pandey, and A. Pitman, "FTIR studies of the changes in wood chemistry following decay by brown-rot and white-rot fungi," *International Biodeterioration & Biodegradation*, vol. 52, no. 3, pp. 151-160, 2003.
- [15] X. Yang, Y. Zeng, F. Ma, X. Zhang, and H. Yu, "Effect of biopretreatment on thermogravimetric and chemical characteristics of corn stover by different white-rot fungi," *Bioresource technology*, vol. 101, no. 14, pp. 5475-5479, 2010.
- [16] D. Ciolacu, F. Ciolacu, and V. I. Popa, "Amorphous cellulose—structure and characterization," *Cellulose chemistry and technology*, vol. 45, no. 1, pp. 1, 2011.
- [17] X.-B. Zhao, L. Wang, and D.-H. Liu, "Peracetic acid pretreatment of sugarcane bagasse for enzymatic hydrolysis: a continued work," *Journal of Chemical Technology & Biotechnology*, vol. 83, no. 6, pp. 950-956, 2008.
- [18] S. Kim, and M. T. Holtzapfel, "Effect of structural features on enzyme digestibility of corn stover," *Bioresource Technology*, vol. 97, no. 4, pp. 583-591, 2006.
- [19] Z. Gao, T. Mori, and R. Kondo, "The pretreatment of corn stover with *Gloeophyllum trabeum* KU-41 for enzymatic hydrolysis," *Biotechnol Biofuels*, vol. 5, no. 1, pp. 1-11, 2012.
- [20] T. Jeoh, C. I. Ishizawa, M. F. Davis, M. E. Himmel, W. S. Adney, and D. K. Johnson, "Cellulase digestibility of pretreated biomass is limited by cellulose accessibility," *Biotechnology and Bioengineering*, vol. 98, no. 1, pp. 112-122, 2007.
- [21] R. Millati, S. Syamsiah, C. Niklasson, M. Nur Cahyanto, K. Lundquist, and M. J. Taherzadeh, "Biological pretreatment of lignocelluloses with white-rot fungi and its applications: a review," *BioResources*, vol. 6, no. 4, 2011.
- [22] F.-h. Sun, J. Li, Y.-x. Yuan, Z.-y. Yan, and X.-f. Liu, "Effect of biological pretreatment with *Trametes hirsuta* yj9 on enzymatic hydrolysis of corn stover," *International Biodeterioration & Biodegradation*, vol. 65, no. 7, pp. 931-938, 10//, 2011.
- [23] M. Kurakake, N. Ide, and T. Komaki, "Biological pretreatment with two bacterial strains for enzymatic hydrolysis of office paper," *Current microbiology*, vol. 54, no. 6, pp. 424-428, 2007.
- [24] P. Singh, A. Suman, P. Tiwari, N. Arya, A. Gaur, and A. Shrivastava, "Biological pretreatment of sugarcane trash for its conversion to fermentable sugars," *World Journal of Microbiology and Biotechnology*, vol. 24, no. 5, pp. 667-673, 2008.
- [25] D. Deswal, R. Gupta, P. Nandal, and R. C. Kuhad, "Fungal pretreatment improves amenability of lignocellulosic material for its saccharification to sugars," *Carbohydrate Polymers*, vol. 99, no. 0, pp. 264-269, 1/2/, 2014.
- [26] M. R. L. T. A. Hsu, and G. T. Tsao, "Alcohol from cellulose," *Chemical Technology*, vol. 10, no. 5, pp. 315-319, 1980.
- [27] J. Ramos, T. Rojas, F. Navarro, F. Dávalos, R. Sanjuán, J. Rutiaga, and R. A. Young, "Enzymatic and Fungal Treatments on Sugarcane Bagasse for the Production of Mechanical Pulps," *Journal of Agricultural and Food Chemistry*, vol. 52, no. 16, pp. 5057-5062, 2004/08/01, 2004.
- [28] M. Galbe, and G. Zacchi, "Pretreatment: The key to efficient utilization of lignocellulosic materials," *Biomass and Bioenergy*, vol. 46, no. 0, pp. 70-78, 11//, 2012.