

Study on Microbial Pretreatment for Enhancing Enzymatic Hydrolysis of Corncob

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Abstract—The complex structure of lignocellulose leads to great difficulties in converting it to fermentable sugars for the ethanol production. The major hydrolysis impediments are the crystallinity of cellulose and the lignin content. To improve the efficiency of enzymatic hydrolysis, microbial pretreatment of corncob was investigated using two bacterial strains of *Bacillus subtilis* A 002 and *Cellulomonas* sp. TISTR 784 (expected to break open the crystalline part of cellulose) and lignin-degrading fungus, *Phanerochaete sordida* SK7 (expected to remove lignin from lignocellulose). The microbial pretreatment was carried out with each strain under its optimum conditions. The pretreated corncob samples were further hydrolyzed to produce reducing glucose with low amounts of commercial cellulase (25 U·g⁻¹ corncob) from *Aspergillus niger*. The corncob samples were determined for composition change by X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and scanning electron microscope (SEM). According to the results, the microbial pretreatment with fungus, *P. sordida* SK7 was the most effective for enhancing enzymatic hydrolysis, approximately, 40% improvement.

Keywords—Corncob, Enzymatic hydrolysis, Microorganisms, Pretreatment.

I. INTRODUCTION

WORLDWIDE use of ethanol as an alternative fuel has been steadily increasing because of the main problems of petroleum shortage and environmental concern. The biomass conversion to ethanol generally consists of the hydrolysis of biomass into reducing sugars and the fermentation of reducing sugars to ethanol [1]. Different kinds of biomass have been used as a feedstock which can be classified into three categories; simple sugar, starch, and lignocellulosic materials [2]. Present processes are likely to use edible plant materials like sugar and starch which are easily hydrolyzed; however, they are in human food chain, affecting food supply and price [3], [4]. To overcome the food conflict problem, lignocellulose biomass like agricultural residues is considered to be a promising alternative feedstock for sugar production.

Thailand is an agricultural country with abundant crop

products, such as corn, sugarcane, rice, etc. Consequently, large amounts of agricultural residues associated with the crop production can be a potential resource for producing energy and other more valuable chemicals. Corncob residue around 1.13 million ton is produced each year [5]. Some portion is used as animal feed, but the excess remaining residues still leads to an environmental problem. Hence, a use of agricultural residues for sugar production is of great interest. The contents of cellulose and hemicelluloses in corncob are very high and vary with species, growing area, season, and so on. Commonly, the main composition of corncob is consisted about 45% cellulose, 35% hemicellulose, and 15% lignin [6].

The complex structure of cellulose, resulted from lignin coverage and rigid crystallinity of cellulose, is responsible for low sugar yield [7]. Therefore, a pretreatment is needed to reduce the biomass recalcitrance by breaking lignin seals and disrupting the crystalline structures of cellulose [8]. Compared with the other pretreatment methods (e.g. diluted acid, alkali extraction, steam explosion, and hydrothermolysis), microbial pretreatment of lignocellulosic materials is an environmentally friendly process in aspects of no severe chemicals, less energy input, no requirement for pressurized and corrosion-resistant reactors, and less inhibition to fermentation [9]. Microbes used for pretreatment of lignocellulosic materials can be both fungal and bacterial species. White rot fungi have been receiving extensive attention for biodelignification of lignocellulosic biomass but several weeks to months are generally needed to obtain a high degree of lignin degradation [8], [10], [11]. In 2007, Kurakake and coworker studied bacterial pretreatments of office paper and found that the sugar recovery up to 94% was achieved after 4 d. They explained that the strains acted as an endoglucanase, to hydrolyze the amorphous areas randomly [12]. For bacterial pretreatment on corncobs has not yet been reported.

In this study, the enhancement of enzymatic hydrolysis by microbial pretreatment was carried out using both fungus and bacterium. The corncob samples were characterized in order to correlate the enzymatic hydrolysis activity to the structural changes as a result from the pretreatment.

II. EXPERIMENTAL

A. Materials and Microbial Strains

Corncob collected from Kanchanaburi province, Thailand, was dried overnight at 105°C. Dried corncob was milled and sieved to the size ranges of 40–60 mesh (0.25–0.42 mm) and stored in an air tight container at room temperature before use. Bacterial pretreatment of corncob was performed by two cellulose-hydrolyzing bacteria including *Bacillus subtilis*

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A002 isolated from Thai higher termites, *Microcerotermes* sp., by our research group [13] and *Cellulomonas* sp. TISTR 784 obtained from sugarcane field provided by Thailand Institute of Scientific and Technological Research. Both studied bacteria were activated in a 65 modified DSMZ broth medium and were then maintained on a 65 modified DSMZ agar medium at 4°C. The white-rot fungi, *Phanerochaete sordida* SK7 used for the pretreatment study was kindly provided by Plant Biomass Utilization Research Unit, Chulalongkorn University, Thailand. The medium and all equipments were sterilized in an autoclave at 121°C for 15 min. Distilled water was used for preparing all solutions.

B. Bacterial Pretreatment

For bacterial inoculation, a 65 modified DSMZ nutrient broth (5 g·L⁻¹ Carboxymethyl cellulose (CMC), 4 g·L⁻¹ yeast extract, and 10 g·L⁻¹ malt extract), pH 7.2 was prepared for culturing *B. subtilis* A 002, and a medium containing 5 g·L⁻¹ CMC, 0.5 g·L⁻¹ yeast extract, 1 g·L⁻¹ NaNO₃, 1 g·L⁻¹ K₂HPO₄, 1 g·L⁻¹ KCl, 0.5 g·L⁻¹ MgSO₄, and 1 g·L⁻¹ glucose at pH 7.2 was prepared for *Cellulomonas* sp. TISTR 784. Each pure colony was cultured in a 250 mL Erlenmeyer flask containing 50 mL of each medium. The culturing flask was incubated at 37°C in a shaking incubator at 180 rpm for 12 h. Then, an inoculum (10⁷ CFU·mL⁻¹) was transferred into a 1 L bottle containing 450 mL of each medium with 5 g of the 40–60 mesh corncobs for pretreatment. The bacterial pretreatment was carried out in the medium without CMC or glucose and incubated at 37°C in a shaking incubator at 180 rpm for 3 d. After that, the pretreated samples were washed with 200 mL DI water and dried until constant weight for weight loss determination and composition analysis.

C. Fungal Pretreatment

The tropical white-rot fungus *P. sordida* SK7 was cultured on a malt extract (ME) agar plate, containing 30 g·L⁻¹ malt extract, 5 g·L⁻¹ mycological peptone, 10 g·L⁻¹ glucose, and 16 g·L⁻¹ agar, at 30°C for 3-5 d until the mycelium fully grew inside the petri dish. Twenty mycelium blocks (10 mm in Diameter) from the pre-cultured fungus were inoculated in a 250 mL Erlenmeyer flask containing 200 mL of ME broth at room temperature with static conditions for 10 d. The grown mycelium mat was aseptically filtered and washed with sterile distilled water and homogenized by homogenizer (Omni TH) for three 20-s cycles and adjusted the mycelium suspension to be 3.5 mg·L⁻¹ on dry basis. The fungal pretreatment was carried out in solid state, following the method published with some modification [10], [11]. A quantity of 5 gram of the dried corncob was placed in a 250 ml Erlenmayer flask and supplemented with 0.5% corn steep liquor then conditioned with distilled water. The mixture was subjected to sterilization at 121°C for 15 min and cooled prior to inoculation. Each flask was loaded with mycelium suspension on the top of the substrate corresponding to a fungal mycelium (mg): corncob (g) ratio of 1:2. The pretreatment was carried out at 30°C with static conditions for 20 d. After that, the pretreated samples were washed with 200 ml DI water and dried until constant

weight for weight loss determination and composition analysis [14].

D. Composition Analysis

For chemical composition analysis, corncob samples were analyzed for cellulose, hemicelluloses, lignin, and extractives. The amount of extractives was determined from the weight loss from solvent extraction using 60 mL acetone per gram of a dried corncob sample at 90°C for 2 h and drying at 105°C. The hemicellulose content was represented by the weight loss from the alkaline dissolution step. A 10 mL of a 0.5 M of sodium hydroxide (NaOH) solution was added to 1 g of extractive-free dried biomass, and the mixture was held at 80°C for 3.5 h. After that, the mixture was washed several times by DI water and dried to a constant weight. To determine the lignin fraction, 30 mL of a 98% sulfuric acid (H₂SO₄) was added to the residue after the alkaline dissolution step, and the mixture was held at ambient temperature for 24 h and boiled at 100°C for 1 h. The mixture was filtered, and then the residue was washed with distilled water until the sulfate ion in the filtrate was undetectable (via titration of a 10% barium chloride solution). Finally, the remaining residue was dried at 105°C until constant weight. The weight of the dried residue was recorded as the lignin content. The cellulose content was calculated by the weight difference of the remains residues by the NaOH and H₂SO₄ extractions [15], [16]. The elemental compositions of samples were also determined by a CHNS/O analyzer (Leco, TruSpec® Elemental Determinator). All samples were assayed in triplicated.

E. Enzymatic Hydrolysis Experiments

The cellulase from *Aspergillus niger* (Sigma Chemical Co.) with the CMCase activities of 0.35 U·mg⁻¹ (following Ghose's method) was used to hydrolyze both untreated and pretreated corncob samples [17]. The enzymatic hydrolysis was carried out with 1% corncob loading and 25 U of cellulase in 100 ml of a 50 mM acetate buffer solution, pH 4.8. A 0.01% sodium azide was added to prevent the microorganism contamination [18]. The hydrolytic mixture was incubated in a shaking incubator at 180 rpm and 37°C for 3 d with periodically sample collecting.

F. Sugar Analysis

The supernatant of each sample taken during the hydrolysis experiments after centrifugation at 8000 rpm for 3 min was filtrated through a 0.22 µm nylon filter and used for sugar analysis by high-performance liquid chromatography (HPLC) equipped with a refractive index detector (Model 6040 XR, Spectra-Physics, USA) and Aminex HPX- 87H column, (Bio-Rad Lab, USA). A 0.005 M H₂SO₄ solution was used as a mobile phase at a flow rate of 0.6 mL·min⁻¹ and a column temperature of 65°C.

G. Crystallinity Measurement

The crystallinity structures of the untreated and pretreated corncob samples were determined by X-ray diffraction (XRD). The dried samples were scanned and recorded by using a Rigaku X-Ray Diffractometer (RINT-2200) with a Ni

filter and Cu K α radiation (1.5406 Å) generated at 30 mA and 40 kV. A scan speed of 5° (2 θ) · min⁻¹ with a scan step of 0.02 (2 θ) was used for the continuous run in 5 to 50° (2 θ) range. Crystallinity index (%) was calculated by the following equation [19]:

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

where I_{002} is the crystalline peak of the maximum intensity at 2 θ between 22° and 23° and I_{am} is the amorphous peak of the minimum intensity at 2 θ between 18° and 19°.

H. Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) analysis was used for analyzing the differences of crystallinity and chemical structure of corncob sample before and after microbial pretreatment. The FTIR spectra of untreated corncob and pretreated corncobs were measured by direct transmittance using the KBr pellet technique. Spectra were recorded using a Nicolet Nexus 670. All the spectra are measured at 100 scans per sample with spectral resolution of 4 cm⁻¹, in the frequency range of 4000–400 cm⁻¹ [20].

I. Morphology Examination

The morphological changes of corncob entities due to the hydrolysis process were observed using a scanning electron microscope (SEM), Hitachi/S-4800.

III. RESULTS AND DISCUSSION

A. The Change of Corncob Composition

The chemical composition of corncob is generally different, depending on breeds, growing area, season, and also analysis method [21]. In this study, corncob was analyzed on dried weight basis. The original corncob sample used in this study consists of 43.64% cellulose, 33.59% hemicellulose, 12.23% lignin, and 10.55% extractives, as shown in Table I. It had a quite high portion of cellulose with lower contents of hemicellulose and lignin— inhibitors of the cellulolytic hydrolysis—, compared with other agricultural residues [6]. After the microbial pretreatment with different strains, the weight losses of samples during pretreatment with *B. subtilis* A 002, *Cellulomonas* sp. TISTR 784, and *P. sordida* SK 7 were 10.73%, 10.93%, and 7.68%, respectively. The results showed that the bacterial pretreatment which was liquid state caused higher weight loss than the fungal pretreatment which was a semi-solid state. After the fungal pretreatment with the white rot fungus, *P. sordida* SK 7, at 30°C for 20 d, the lignin content (10.08%) was significantly lower than that of the untreated corncob (12.23%) whereas the extractives fraction also increased remarkably from 10.55% to 18.31%. The results imply that the lignin was substantially degraded to low molecular phenolic compounds by the ligninase of the white rot fungi. This is consistent with the result of Lee and coworker who reported that after fungal pretreatment, the content of extractives increased because of degradation products of carbohydrates and lignin [22]. The cellulose

content decreased from 43.64% of the untreated corncob to 38.47% after the fungal pretreatment indicating that it might be from the cellulose decomposition. Normally, the white rot fungi grow on cellulose fibers and produce hyphal sheaths causing cellulose disruption [23]. As a result, the hemicellulose attaching to lignin after lignin removal can be easily degraded. However, this hemicellulose fraction was found to be insignificantly different from the untreated corncob after all pretreatment methods. For the bacterial pretreatment with both *B. subtilis* A 002 and *Cellulomonas* sp. TISTR 784 at 37°C for 2 d, the cellulose content slightly decreased, correlating with the increase of crystallinity index. The results indicating that the amorphous fraction of cellulose was degraded during the pretreatment. There are consistent with the study of Kurakake and coworker who reported that bacterial pretreatment showed an influence on the amorphous area of cellulosic materials [12].

TABLE I
CHEMICAL COMPOSITION OF UNTREATED AND PRETREATED CORNCOB SAMPLES

Microbial strain	Weight loss (%)	Corncob composition (%)			
		Cellulose	Hemicellulose	Lignin	Extractives
Untreated	-	43.64	33.59	12.23	10.55
A 002	10.73	43.03	34.28	14.93	7.76
TISTR 784	10.93	42.20	35.00	17.03	5.77
SK 7	7.68	38.47	33.14	10.08	18.31

B. Enzymatic Glucose Production Abilities

The cellulase (25 U·g⁻¹ corncob) from *Aspergillus niger* (Sigma Chemical Co.) was used to hydrolyze untreated and pretreated corncob samples in 100 mL of a 50 mM acetate buffer solution at pH 4.8 in order to compare the abilities of glucose production. The efficiencies of microbial pretreatments by different strains were evaluated by the amount of produced glucose obtained from the enzymatic hydrolysis step. No other soluble sugar, except glucose, formed during the hydrolysis was observed. The profiles of glucose concentrations over a 72 h period are shown in Fig. 1.

For any given microbial pretreatment, the glucose concentration from the enzymatic hydrolysis of corncob increased with a fast rate within the first 6 h and continually increased with a decreasing rate until 24 h. After that, it still increased with a constant rate. The results can be explained by the fact that the enzymatic hydrolysis of corncob is a heterogenous catalytic reaction, which depends directly on the available interfacial surface area. At the beginning of the reaction, there is more possibility of the soluble enzyme to adsorb on the more vacant site of corncob surface to undergo the reaction, leading to the decrease in available surface. Afterwards, the reaction takes place with the decreasing available surface, resulting in a slower glucose production rate. This result is in a good agreement with a report of Highina and coworker [24].

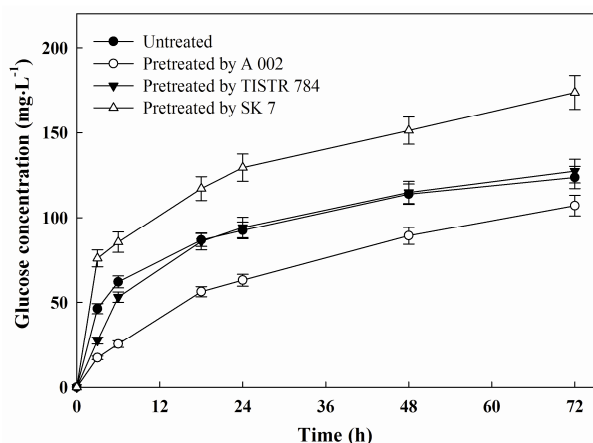


Fig. 1 Glucose concentrations from enzymatic hydrolysis of untreated and pretreated corncob

The enzymatic hydrolysis of the untreated corncob provided $124 \text{ mg} \cdot \text{L}^{-1}$ of glucose after 72 h. The enzymatic hydrolysis with the fungal pretreatment using *P. sordida* SK 7 provided the highest glucose concentration ($174 \text{ mg} \cdot \text{L}^{-1}$). It can improve enzymatic hydrolysis up to 40% compared with untreated. The increase in glucose yield indicates the decrease in the recalcitrance of the enzymatic hydrolysis, which results from the altering of the corncob characteristics during the pretreatment with the white rot fungi. These characteristics might include an increase in adsorption capacity to cellulase and a decrease in lignin content [6], [25]-[28]. For the purpose of using *B. subtilis* A 002 for the bacterial pretreatment of corncob in order to hydrolyze partially the crystalline cellulose fraction into amorphous form; however, it provided the negative result of glucose production after the enzymatic hydrolysis. The glucose produced from pretreated corncob by *B. subtilis* A002 was lower than the untreated corncob ($107 \text{ mg} \cdot \text{L}^{-1}$). It can be explained by the XRD result which showed the increase of crystallinity index of corncob after pretreatment (more details on the following section). The result imply that the pretreatment using *B. subtilis* A 002 major acted on amorphous part and still leaved the crystalline part which was difficult to be hydrolyzed. This is the reason why the pretreated corncob provided glucose less than the untreated corncob, which possessed more amorphous cellulose, did. This situation also occurred with pretreated corncob using *Cellulomonas* sp. TISTR 784. However, it was possible that less activity of *Cellulomonas* sp. TISTR 784 made glucose insignificantly differ from the untreated one ($127 \text{ mg} \cdot \text{L}^{-1}$).

C. Crystallinity Measurement by XRD

The structure of corncob contains crystalline portion of cellulose and amorphous portion of hemicellulose and lignin. Normally, the latter portion makes the access of cellulase enzymes to cellulose difficult. Thus, the transformation from crystalline to amorphous structure during a pretreatment can improve the subsequent step of enzymatic hydrolysis efficiency [29], [30]. In order to observe the change in

corncob structure, XRD was used to investigate the crystallinity after microbial pretreatment and enzymatic hydrolysis. Fig. 2 shows the X-ray diffractograms of untreated and microbial pretreated corncob samples. The crystallinity index was calculated from the intensity of the crystalline peak obtained at 2θ between 22° and 23° and the amorphous peak obtained between 18° and 19° . The diffractograms of the pretreated corncob with *P. sordida* SK 7 exhibited that the crystalline peak was significantly decreased compared with the others.

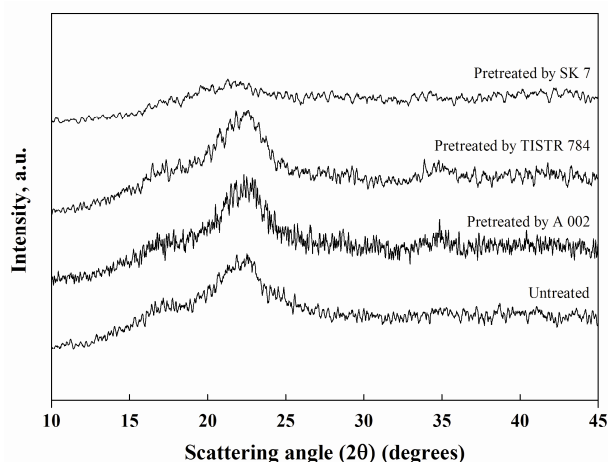


Fig. 2 X-ray diffractograms of untreated and pretreated corncob samples

In Table II, the pretreated corncob using fungus, *P. sordida* SK 7 provided the greatest effect on crystallinity change. Its crystallinity index decreased from 57.14% to 54.35%. Sripat reported that *P. sordida*, SK 7 had the ability to produce ligninase enzyme—Ligninase peroxidase and Laccase [14]. Ligninase can degrade lignin, causing the increase of pore sizes and accessible surface area to cellulase [8]. Therefore, the crystalline cellulose after the lignin removal becomes less crystallinity. Furthermore, the degradation of cellulose also results in the decrease in crystallinity index. After pretreatment using two bacterial strains of *B. subtilis* A 002 and *Cellulomonas* sp. TISTR 784, the crystallinity index was up to 68.01% and 58.04%, respectively. Both *B. subtilis* and *Cellulomonas* sp. have been reported about the production of cellulose-hydrolyzing enzyme [13], [31]-[34]. Therefore, we found that the amorphous portion was preferentially removed during the pretreatment step, confirmed by the decrease of cellulose content (Table I). After the enzymatic hydrolysis of any given pretreated corncob samples, the crystallinity indices decreased. On the other hand, the untreated corncob shows the increase in crystallinity index after the enzymatic hydrolysis, suggesting that the cellulase can preferentially hydrolyzed on the amorphous cellulose.

TABLE II
CRYSTALLINITY INDEX OF CORNCOB AFTER MICROBIAL PRETREATMENT AND ENZYMATIC HYDROLYSIS

Microbial strain	Crystallinity (%) of corncob	
	Post-pretreatment	Post-enzymatic hydrolysis
Untreated	57.14	66.02
A 002	68.01	64.07
TISTR 784	58.04	55.74
SK 7	54.35	53.22

D. The Change in Chemistry of Pretreated Corncob by FTIR

The Fourier transform infrared (FTIR) spectroscopic results of microbial pretreated corncob samples are shown in Fig. 3. The analysis of corncob is based on spectra band absorptions for lignocelluloses [20], as shown in Table III. The stronger bands of hydrogen bonded (O-H) stretching absorption at 3400 cm^{-1} (1) and C-H stretching absorption at 2912 cm^{-1} (2) was found in the pretreated corncob samples compared with untreated corncob. When extractives or lignin were removed after pretreatment, the portion of cellulose and hemicellulose increased, resulting in these stronger bands. The existence of band at 1735 cm^{-1} (3) in all pretreated corncobs represented the unconjugated C=O in xylan or hemicellulose which still remained after the microbial pretreatment, suggesting that the microbial pretreatment step could not break down the hemicellulose in the studied corncob. The intensities of absorbance bands at 1113 cm^{-1} (13) of aromatic skeletal and C-O stretch and 1601 cm^{-1} (5) of aromatic skeletal in lignin obviously decreased after the fungal pretreatment by *P. sordid* SK 7, indicating that the selective removal of the lignin occurred by this fungal strain.

E. Surface Morphology by SEM

The smooth surface was observed at the untreated corncob, as shown in Fig. 4 (a). Interestingly, the surface morphology was obviously changed to be rough and porous after the all microbial pretreatments of corncobs. Among all pretreatment methods, the fungal pretreatment shows the roughest surface with fungal fibers remaining on surface (Fig. 4 (d)). The change in surface morphology confirms microbial action on corncob as mention before.

TABLE III
KEY FTIR ABSORPTIONS FOR LIGNOCELLULOSE [20]

Peak number	Key spectra	Wave number (cm^{-1})
1	A strong hydrogen bonded (O-H) stretching absorption	3400
2	A prominent C-H stretching absorption	2912
3	Unconjugated C=O in xylans (hemicellulose)	1735
4	Absorbed O-H and conjugated C-O	1634
5	Aromatic skeletal in lignin	1601
6	Aromatic skeletal in lignin	1516
7	C-H deformation in lignin and carbohydrates	1463
8	C-H deformation in lignin and carbohydrates	1429
9	C-H deformation in cellulose and hemicellulose	1378
10	C-H vibration in cellulose and C-O vibration in syringyl derivatives	1325
11	Guaiacyl ring breathing, C-O stretch in lignin and C-O linkage in guaiacyl aromatic methoxyl groups	1257
12	C-O-C vibration in cellulose and hemicellulose	1166
13	Aromatic skeletal and C-O stretch	1113
14	C-O stretch in cellulose and hemicellulose	1050
15	C-H deformation in cellulose	895

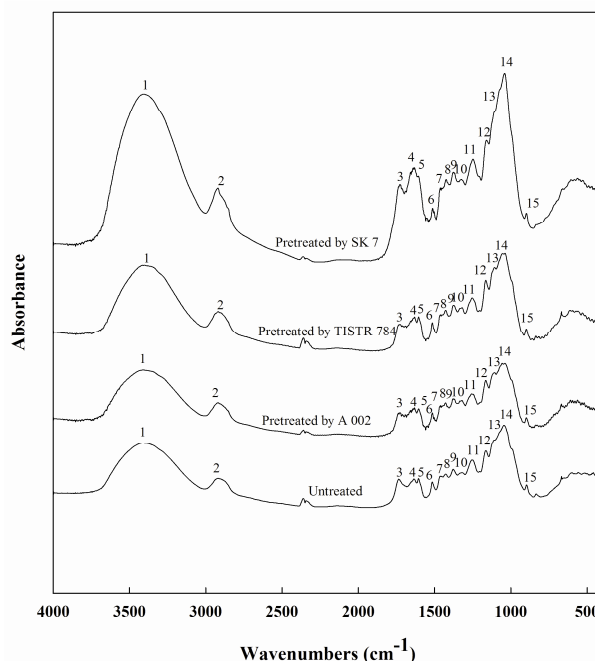


Fig. 3 FTIR spectra of untreated and pretreated corncob samples

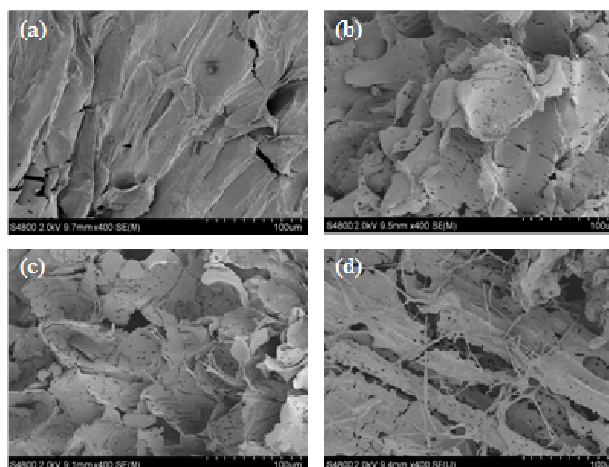


Fig. 4 Scanning electron micrographs of the 40–60 mesh corncob surfaces (a) untreated corncob; (b) pretreated with *B. subtilis* A 002; (c) pretreated with *Cellulomonas* sp. TISTR 784; and (d) pretreated with *P. sordida* SK 7

IV. CONCLUSIONS

Microbial pretreatment is the environmentally friendly process for lignocellulose pretreatment before enzymatic hydrolysis because it can be carried out at mild conditions and no hazardous chemical requirement. The results showed that the microbial pretreatment using lignin degrading fungus, *P. sordid* SK7 on corncob was an effective method in improving enzymatic hydrolysis accessibility (approximately, 40% improvement) compared with using cellulase producing bacteria, *B. subtilis* A002 and *Cellulomonas* sp. TISTR 784. Due to *P. sordid* SK7 can produce ligninase for degrading the

lignin which is the major impediment in enzymatic hydrolysis. However, fungal pretreatment is time consuming process compared with bacterial pretreatment. More extensive study need to be performed to compare in aspect of economic feasibility.

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