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Development of Efficient Fungal Biomass-Degrading Enzyme Mixtures for Saccharification of Local Lignocellulosic Feedstock

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***Abstract*—**Conversion of lignocellulosic biomass is the basis process for production of fuels, chemicals and materials in the sustainable biorefinery industry. Saccharification of lignocellulosic biomass is an essential step which produces sugars for further conversion to target value-added products e.g. bio-ethanol, bio-plastic, γ-valerolactone (GVL), 5-hydroxymethylfuroic acid (HMF), levulinic acid, etc. The goal of this work was to develop an efficient enzyme for conversion of biomass to reducing sugar based on crude fungal enzyme from *Chaetomium globosum* BCC5776 produced by submerged fermentation and evaluate its activity comparing to a commercial *Acremonium* cellulase. Five local biomasses in Thailand: rice straw, sugarcane bagasse, corncobs, corn stovers, and palm empty fruit bunches were pretreated and hydrolyzed with varying enzyme loadings. Saccharification of the biomass led to different reducing sugar levels from 115 mg/g to 720 mg/g from different types of biomass using cellulase dosage of 9 FPU/g. The reducing sugar will be further employed as sugar feedstock for production of ethanol or commodity chemicals. This work demonstrated the use of promising enzyme candidate for conversion of local lignocellulosic biomass in biorefinery industry.

***Keywords*—**Biomass, Cellulase, *Chaetomiun glubosum*, Saccharification.

I. Introduction

NOWADAYS the crisis of rising prices in crude oil particularly for industrial and transportation sectors due to rapidly increasing fuel demands and decreasing oil supplies leads to the shortage conventional energy resources (coal, oil and gas). In addition, consumption of petroleum also emits excessive CO_2 into the atmosphere, resulting in environmental concerns such as global climate change [1]. Many countries have concerned about energy and environment issues and thus finding alternative clean energy technology is encouraged.

Recently, one of the most attractive approaches is to utilize abundant lignocellulosic materials instead of conventional fuels. Production of fuels, chemicals and materials based on biomass feedstock is an interesting alternative since lignocellulose is renewable, clean, inexpensive and no harmful to environment; besides, it does not negatively affect to human food supply.

Lignocellulosic biomass is biological materials derived from living organisms including agricultural wastes, wood wastes, and forestry residues. It consists mainly of three different types of polymers i.e. cellulose, hemicellulose and lignin which are associated with each other [2].

Hydrolysis of lignocellulosic biomass into sugars comprises two steps: the pretreatment of biomass and the enzymatic saccharification of the pretreated biomass into sugars for further conversion. Based on current technologies, the cost of ethanol production is relatively high and the main challenges are the low yield and high cost of the hydrolysis process. Therefore, optimization of hydrolysis process is thus necessary for their efficient conversion and production of the target products.

Zhong et al., 2009 [3] reported optimization of enzymatic hydrolysis from AFEX-treated rice straw. They found that hydrolysis efficiency of lignocellulosic biomass increased when combining of enzymes such as cellulase, xylanases and pectinases. Glucan and xylan conversion into monomeric sugars reached 81.7% and 75.8% when using enzyme loading 15 FPU/g glucan. In addition, Kun et al., 2009 [4] also studied enzymatic saccharification of pretreated-rice straw. The result verified that conversion of cellulose and hemicelluloses into fermentable sugar was 73.96%.

Zhao et al., 2011 [5] presented enzymatic saccharification of alkali/peracetic acid (PAA)-pretreated bagasse. The effects of initial solid substrate, cellulase loading and addition of supplemented β-glucosidase were investigated in their work. The result showed that enzymatic conversion of glucan reached 80% after 24 h incubation period when enzyme loading was 10 FPU/g solid.

Buaban et al., 2010 [6] studied bioethanol production from ball milled bagasse using on-site produced fungal enzyme cocktail and fermentation of xylose by *Pichia stipitis*. The sugar yield (%) from enzymatic hydrolysis with different enzyme preparations was investigated. It was found that the maximum yield of sugars of 84.0 % glucose and 70.4 % xylose were obtained from combination of the *Penicillium chrysogenum* BCC 4504 cellulase with *Aspergillus flavus* BCC 179 enzyme preparation containing high β-glucosidase and xylanase activities.

The aims of this research are; (1) to convert local lignocellulosic biomass (i.e. sugarcane bagasse, corncobs,

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corn stovers, rice straw and palm empty fruit bunches) into fermentable sugar using enzymatic saccharification and (2) to compare reducing sugar obtained from BCC 5776 crude enzymes preparation with a commercial *Acremonium* cellulase. The work provides a promising alternative fungal enzyme for application in biomass saccharification process in sugar platform biorefinery.

II. Methodology

A. Biomass Preparation

All the biomass used in this work was obtained locally. Sugarcane bagasse was obtained from Phu Kiaew Bioenergy, Chiayapoom, Thailand. Corn cob and corn stovers were obtained from Suwan farm, Nakorn Ratchasrima, Thailand. Rice straw was obtained from a local farm in Ayutthaya, Thailand. Palm empty fruit bunches were obtained from Suksomboon Palm Oil Co., Ltd., Chonburi, Thailand. The biomass was physically processed using a SM2000 cutting mill (Retsch, Haan, Germany) and sieved through a 0.5mm mesh. The pretreated of sugarcane bagasse and palm empty fruit bunches were performed using 2 steps pretreatment. In the first step, 5% (w/v) NaOH was employed and treated at 90ºC (sugarcane bagasse) and 70ºC (palm empty fruit bunches) for 90min. Then, 5% (w/v) peracetic acid (PAA) was introduced at 70ºC for 150min. Corncobs and corn stovers were pretreated with 5% NaOH (w/v) at 90ºC (corncobs) and 80ºC (corn stovers) for 90min. Rice straw was pretreated with 10% (w/v) NaOH at 80ºC for 90min. The pretreated biomass was washed with distilled water until its pH decreased to 7 and was then dried at 60ºC. The chemical compositions of biomass before and after pretreatment were analyzed using the following standard TAPPI methods (1992):α-cellulose (T203 om-83); Klason lignin (T222 om-83); and pentosans (T223 hm-84).

B. Microorganism and Enzyme

A commercial cellulase (i.e. *Acremonium cellulyticus*) was obtained from the Advanced Institute of Industrial Science and Technology (AIST), Hiroshima, Japan. The enzyme activity was 50.5 FPU/ml. *C. globosum* BCC5776 and other fungal isolates were obtained from the BIOTEC Culture Collection (www.biotec.or.th/bcc). The fungi were grown on potato dextrose agar (PDA) plates and maintained at room temperature for 7 days.

C. Incoculum Preparation

The fungal culture was prepared from fungus grown on PDA by excising five agar pieces covered with a profuse mycelial mat using a No. 2 cork borer and inoculated in cellulase production medium containing 50 ml working volume in which 2% AVICEL® and 1% palm empty fruit bunches as carbon sources, 1% soybean meal as nitrogen source, 0.1% (v/v) lactose, 50mM potassium phosphate buffer (pH 5.8) and distilled water. The optimized medium was maintained at 30ºC for 6 days with rotary shaking at 200 rpm.

D. Fermentation Experiment

Fermentation run was carried out on 5-L bioreactor (batch fermenter) containing 3-L working volume. The optimized production medium contained 60g AVICEL®, 30g pretreated palm empty fruit bunches, 30g soybean meal, 300ml 1% (v/v) lactose, 150ml 1M potassium phosphate buffer (pH 5.8) and distilled water. The cultures were also incubated under the identical conditions, as mentioned above for 6 days.

E. Crude Fungal Enzyme Extract Preparation

For the preparation of crude enzyme extracts, the cells were separated by centrifugation at 5,000 rpm and the supernatants were filter–sterilized through a 0.2 µm Supor®-200 membrane (Pall Corp, Ann Arbor, MI). The selected crude enzyme extracts were concentrated by ultra filtration on a Minimate™ tangential flow filtration (TFF) system using a Minimate ™ TFF capsule with a 10-kDa MWCO membrane (Pall Corp, Ann Arbor, MI). The enzyme solutions were kept at 4ºC until used.

F. Enzyme Assays

The polysaccharide-degrading activities were analyzed based on the amount of released reducing sugars using the 3,5-dinitrosalicylic acid (DNS) method [7]. For the filter paper cellulase (FPase) analysis, the 3.5-ml reactions contained an appropriate dilution of the enzyme in 100mM sodium acetate buffer (pH 5.5) and a piece of 1 ×1.5cm Whatman No. 1 filter paper as a substrate. The reactions were incubated at 50ºC for 60min. The carboxymethyl cellulase (CMCase) and xylanase activities were assayed using 1% carboxymethyl cellulose and 1% birchwood xylan as the substrates, respectively. The 3-ml reactions were incubated at 50 ºC for 30min. The mannanase activities was assayed using 0.5% locust beangum as the substrate, the total reaction volume of 3-ml was performed in 100mM sodium acetate buffer (pH 5.5) and incubated at 50ºC for 30min. The amylase and pectinase activities were also assayed using 1% soluble starch and 0.5% pectin from citrus peel as the substrates, respectively, according to the above method and incubated at 50ºC for 30min. The amount of reducing sugars was determined via absorbance measurements at 540nm based on the standard curves prepared using the corresponding sugars. One unit of enzyme activity is defined as the amount of enzyme required releasing 1µmol of glucose/mannose or xylose per min under the assay conditions. To measure the β-glucosidase and β-xylosidase activities, the reaction mixture contained an appropriate amount of enzyme solution, 100mM sodium acetate buffer (pH 5.5), and 0.1% (w/v) p-nitrophenyl-β-D-glucopyranoside (PNPG;Sigma) or p-nitrophenyl-β-D-xylopyranoside (PNPX; Sigma) as a substrate, respectively. After incubation at 50ºC for 60min, the reaction was stopped by the addition of 1M Na_2CO_3. The quantity of p-nitrophenolate was measured spectrophotometrically at 405 nm. One unit of enzyme refers to the amount of enzyme that produces 1µmol p-nitrophenol per min under the assay conditions.

G. Enzymatic Saccharification of Biomass

The hydrolysis reactions were performed in 1.5-ml tubes with a total reaction volume of 1ml, which contained 5% (w/v) pretreated biomass including sugarcane bagasse, corncobs, corn stovers, rice straw and palm empty fruit bunches, 50 mM sodium acetate buffer (pH 5.5), 2% sodium azide, and the indicated amount of enzyme. An equal amount of *Acremonium* cellulase and crude fungal enzyme samples were applied at 3, 6 and 9 FPU/g of substrate. The hydrolysis reactions were incubated at 50ºC with shaking at 200rpm for 72h. The samples were taken to measure reducing sugar 24, 48 and 72h. The reducing sugar yields were determined using the DNS method.

III. Results and Discussion

A. Submerged Fermentation

In the first step, 228 fungal strains from the BIOTEC Culture Collection (BCC) were screened for cellulase production based on filter paper cellulase (FPase) activity level. This fungal strain, *C. globosum,* was cultivated in submerged fermentation containing AVICEL® and palm empty fruit bunches as carbon substrates which were the optimal medium to achieve high level of plant degrading-enzymes according to medium composition optimization study in the laboratory. Furthermore, the enzyme production was up-scaled in a 5-L bioreactor and the concentrated crude fungal enzyme extracts were employed for further study in biomass saccharification. Filter paper activity (FPA) and pH curves of *C. globosum* BCC5776 obtained in 5-L fermenter with working volume of 3 liter under liquid fermentation was shown in Fig. 3. The batch fermentation was carried out using AVICEL® and palm empty fruit bunches as carbon substrates for 6 days. The maximum filter paper cellulase (FPase) activity was 0.40 FPU/ml in the 6th day with a final pH 6.5.The increase of filter paper cellulase activity was found to depend on pH of the medium which corresponded to Kumar, Singh et al., [8]. They reported that pH is an important factor affecting cellulase production. The optimal pH range for CMCase, FPase and β-glucosidase production are 4.5-7.0 for different fungal strains.

B. Compositions of Enzyme Activity Profiles of BCC5776 Enzyme Extracts

The crude enzyme extracts BCC 5776 from the 5-L bioreactor was employed to study the composite cellulolytic and hemicellulolytic activity profiles, compared to the *Acremonium* cellulase. Overall, the native non-concentrated enzyme from the native fungal strain BCC5776 showed relatively lower activities compared to commercial enzymes (Table I and Figs. 1(a)-2(b)); however, they possessed higher relative activities per FPU for downstream activities e.g. β-glucosidase, xylanase and β-xylosidase when compared to the commercial cellulase. *Acremonium* cellulase presented relatively weak β-glucosidase (3.5 U/FPU), xylanase (19.30 U/FPU) and β-xylosidase (0.03 U/FPU) per FPU. The crude fungal enzyme was then concentrated by ultrafiltration and then used to hydrolyze pretreated-lignocellulosic biomass of Thailand.

TABLE I
The Composite Enzyme Activity Profiles of BCC5776 Crude Enzyme Extract

Strain	Activity (U/FPU)							
	CMCase	FPase	β-glucosidase	Xylanase	β-xylosidase	Amylase	Mannanase	Pectinase
Chaetomium globosum (BCC5776)	39.3	1.0	3.6	70.5	0.10	1.3	34.0	2.0

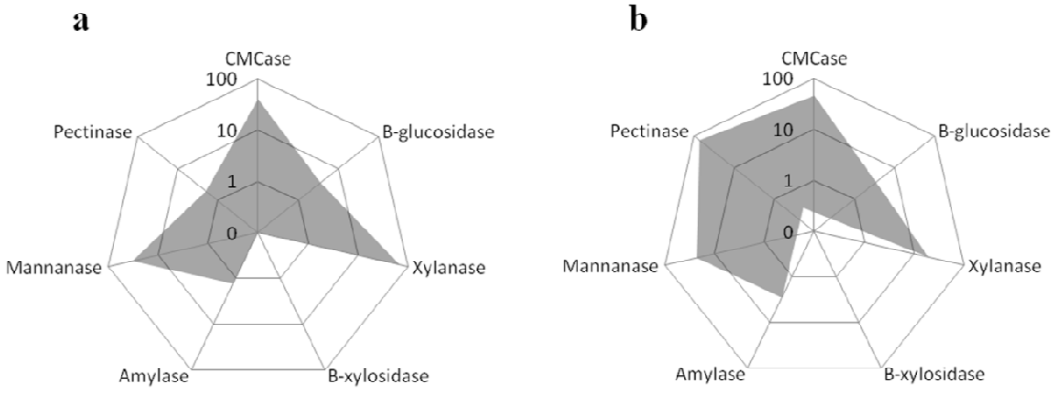

Fig. 1 Composite enzyme activity profiles of BCC5776 enzyme extract and *A. cellulyticus* (commercial cellulase). The Y-axis is in a logarithm scale (log U/FPU). (a) BCC 5776 (b) *A. cellulyticus*

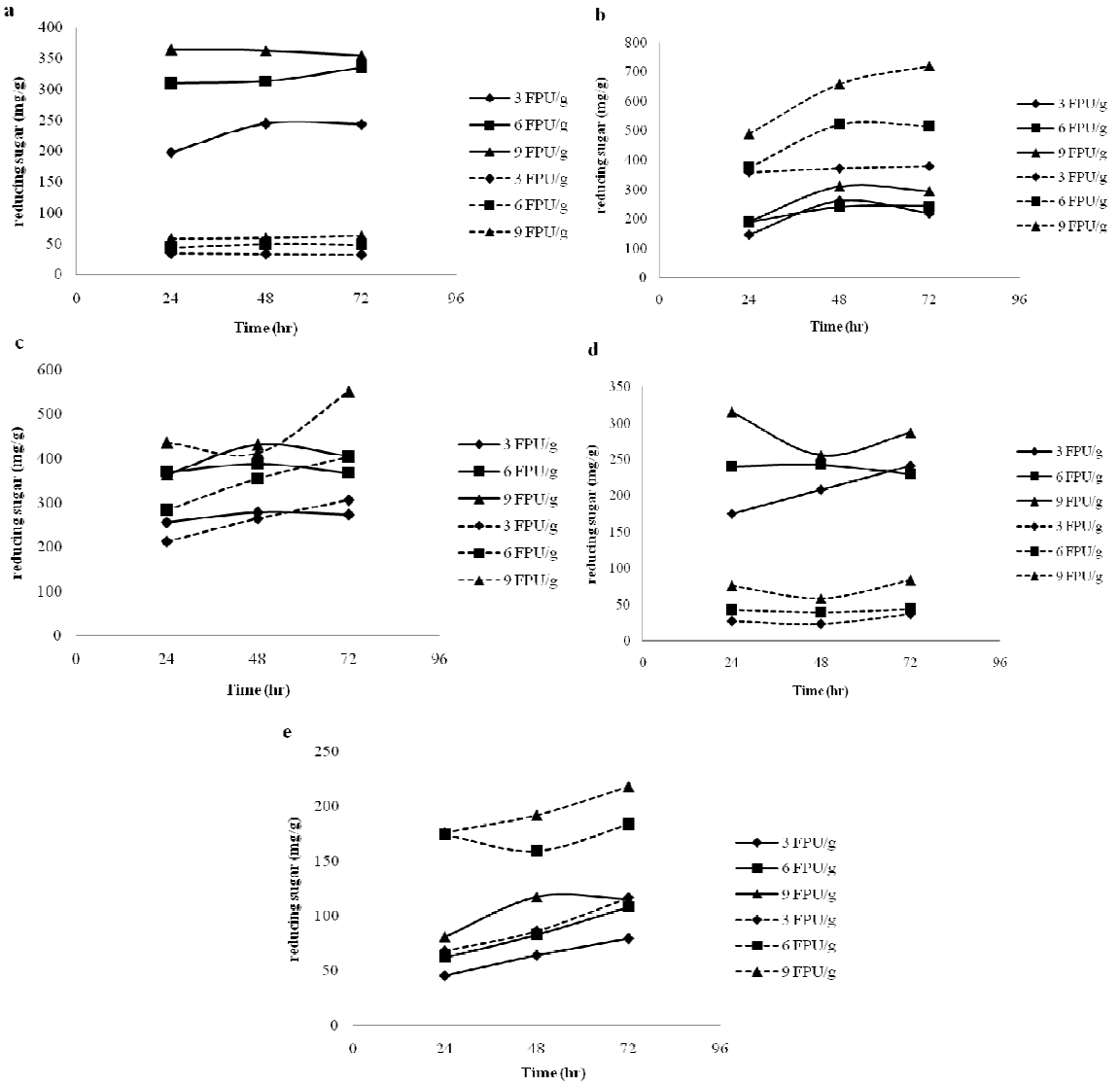

Fig. 2 The reducing sugar yields from the hydrolysis of different types of biomass using different enzyme loading (3 FPU/g, 6 FPU/g and 9 FPU/g) of BCC 5776 concentrated fungal enzyme and commercial cellulase (*A. cellulyticus*). The reactions contained 5% (w/v) pretreated-biomass in 50 mM sodium acetate buffer (pH 5.5) were incubated at 50 ºC for 24 h,48 h and 72 h. (a) rice straw; (b) sugarcane bagasse; (c) corncobs; (d) corn stovers; (e) palm empty fruit bunches (——BCC5776 enzyme; - - - - *A. cellulyticus* cellulase)

C. Biomass Saccharification

Five types of biomass in Thailand (i.e. rice straw, sugarcane bagasse, corncobs, corn stovers and palm empty fruit bunches) were used as the substrates for conversion to sugars using the developed enzyme.

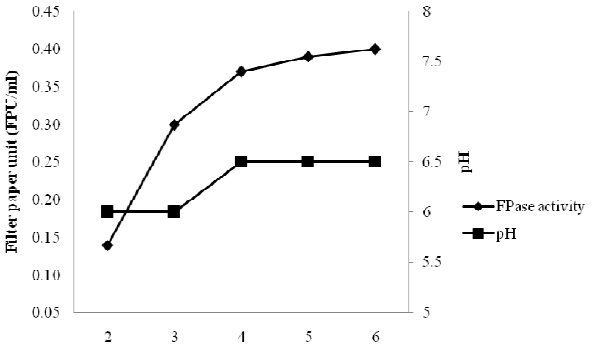

Fig. 3 Filter paper cellulase (FPase) activity and pH profile from batch fermentation of C. glubosum BCC5776 in 5-L fermenter using the optimized medium for 6 days

The results from enzymatic hydrolysis of different pretreated-biomass hydrolysis were shown in Figs. 2(a)-2(e). Increasing sugar was obtained with increasing enzyme dosages for all biomass. Higher reducing sugars were obtained using BCC5776 enzyme compared to *Acremonium* cellulase from hydrolysis of pretreated rice straw and corn stovers. Alkaline pretreated-rice straw was hydrolyzed with different enzyme loadings (3 FPU/g, 6 FPU/g and 9 FPU/g) for 24h, 48h, and 72h using BCC5776 enzyme compared with *Acremonium* cellulase. BCC5776 concentrated fungal enzyme completed hydrolysis of pretreated-rice straw using 6 FPU/g and 9 FPU/g within 48h and gave higher reducing sugar of >300 mg/g biomass, which were higher than those obtained with *Acremonium* cellulase. This is similar for hydrolysis of alkaline-pretreated corn stovers from which >240 mg/g reducing sugar were obtained using BCC5776 higher than that found when *Acremonium* cellulase was used. In contrast, higher saccharification efficiency was obtained using the *Acremonium* cellulase from hydrolysis of bagasse and palm empty fruit bunches. Obviously, in 2 steps pretreated-sugarcane bagasse, the highest reducing sugar of 720 mg/g biomass was obtained with a high dose of *Acremonium* cellulase (9 FPU/g biomass). Palm empty fruit bunches was also pretreated using the same technique as sugarcane bagasse. The result exhibited that hydrolysis of palm empty fruit bunches with lower dose of *Acremonium* cellulase (3 FPU/g) almost produced the same yield of reducing sugar, which was about 80 mg/g biomass, compared to BCC 5776 concentrated enzyme (6 FPU/g). An equal FPU of both BCC 5776 and *Acremonium* cellulase nearly provided the same reducing sugar yield from hydrolysis of pretreated-corncobs.

IV. Conclusion

In this study, enzymatic hydrolysis of five local biomass of Thailand including rice straw, sugarcane bagasse, corncobs, corn stovers and palm empty fruit bunches was studied using the cellulase produced by *C. globosum* BCC5776. The BCC5776 enzyme and the commercial *Acremonium* cellulase showed varying activity on saccharification of the different biomass which reflected differences in composite enzyme activity profiles and properties of the pretreated biomass. Further works will include optimization of the enzyme mixture and enzymatic hydrolysis reaction in order to improve the sugar yield. The work provided a promising enzyme candidate for on-site enzyme production for sugar-platform biorefinery industry.

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