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Ammonia Fiber Expansion (AFEX) Pretreatment, Enzymatic Hydrolysis, and Fermentation on Empty Palm Fruit Bunch Fiber (EPFBF) for Cellulosic Ethanol Production

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Abstract Empty palm fruit bunch fiber (EPFBF), a readily available cellulosic biomass from palm processing facilities, is investigated as a potential carbohydrate source for cellulosic ethanol production. This feedstock was pretreated using ammonia fiber expansion (AFEX) and enzymatically hydrolyzed. The best tested AFEX conditions were at 135 °C, 45 min retention time, water to dry biomass loading of 1:1 (weight ratio), and ammonia to dry biomass loading of 1:1 (weight ratio). The particle size of the pretreated biomass was reduced post-AFEX. The optimized enzyme formulation consists of Accellerase (84 μL/g biomass), Multifect Xylanase (31 μL/g biomass), and Multifect Pectinase (24 μL/g biomass). This mixture achieved close to 90% of the total maximum yield within 72 h of enzymatic hydrolysis. Fermentation on the water extract of this biomass affirms that nutrients solely from the pretreated EPFBF can support yeast growth for complete glucose fermentation. These results suggest that AFEX-treated EPFBF can be used for cellulosic biofuels production because biomass recalcitrance has been overcome without reducing the fermentability of the pretreated materials.

Keywords Cellulosic ethanol · Palm fiber · AFEX · *Saccharomyces cerevisiae* · Biofuel · Pretreatment

Introduction

Lignocellulose, the most abundant organic matter on earth, can be utilized to produce various renewable fuels and chemicals. Unlike conventional fossil-based energy, cellulosic

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biofuels are based on locally produced plant biomass and residues [1, 2]; this reduces geopolitical concerns for liquid fuel supply for many countries. Oil palm is one of the most important crops in Southeast Asian countries, particularly Indonesia and Malaysia, due to its superior oil yield per hectare [3]. Nevertheless, the potential of the lignocellulosic residues from the palm plantation for renewable fuels is virtually untapped. Empty palm fruit bunch fiber (EPFBF), one of the agricultural residues from the oil palm plantation, has alternative uses as a mulch and as a raw material for organic fertilizers [4], but is largely being burned instead of being used productively [5], thus causing environmental pollutions.

Ammonia fiber expansion (AFEX) is a pretreatment method that utilizes concentrated ammonia to overcome the recalcitrance of plant biomass and thereby renders such materials reactive for hydrolytic activities [6]. This pretreatment method has been shown effective on multiple lignocellulosic feedstock such as corn stover [7], switchgrass [8], rice straw [9], and miscanthus [10]. However, the effectiveness of AFEX on agricultural residues from palm plantation is not known.

This report focuses on evaluating the scale of available agricultural residues from palm plantation for cellulosic biofuel production and developing a comprehensive feedstock-to-fermentable sugar conversion technology based on EPFBF and AFEX pretreatment. Specifically, we investigate (1) the condition for AFEX pretreatment on the fiber, (2) enzyme formulation to effectively hydrolyze plant carbohydrate, and (3) ethanol fermentation on the water extract from AFEX-pretreated EPFBF.

Materials and Methods

Empty Palm Fruit Bunch Fiber

Empty bunches were collected from Lenga Palm Oil Industries Sdn. Bhd. (Malaysia), a palm processing factory. These bunches were washed to remove soil and dirt. The washed bunches were defibrated and the fiber lengths were reduced (cut) to 10–15 cm. The bunch fiber was then ground and passed through a screen of 4 mm mesh. The composition of the EPFBF in terms of total glucan, xylan, and arabinan was analyzed based on NREL protocol (LAP-012).

Parametric Exploration of AFEX Pretreatment on EPFBF

The AFEX pretreatment conditions (temperature, moisture content, and particle size) were explored. The details of the explored conditions were as listed in Table 1.

AFEX pretreatment was conducted in a stainless steel Parr reactor. Anhydrous ammonia was preheated and loaded to the reactor which contained EPFBF at the designated water (moisture)

Table 1 Conditions for the parametric exploration for AFEX pretreatment on EPFBF.

Batch no.	Temperature (°C)	Moisture Content (g water/g dry biomass)	Post-AFEX biomass size reduction (0.25-mm screen)	Fixed parameters
1	115	1:1	No	Residence time: 45 min
2	135	1:1	No	Ammonia loading: 1 g NH_3 to 1 g dry biomass
3	155	1:1	No	
4	155	1.5:1	No	Biomass loading per batch: 25 dry gram
5	135	1:1	Yes	

content (Table 1). The initial internal reactor was at 115–120 °C. When needed, the reactor was further heated or maintained at the designated temperature (Table 1) using a heater mantle. The designated temperature was achieved within 3 min after the preheated ammonia was loaded. The residence time (including the heating time, if applicable) of the reaction was 45 min. The pressure was then released and the AFEX-pretreated EPFBF was left under hood to be air-dried overnight. Other operational details were as previously described [11].

Enzymatic Hydrolysis

All enzymatic hydrolyses were conducted in a 20-mL vial at working volume of 15 mL at pH 4.8, 50 °C, 200 rpm agitation. Monomeric glucose and xylose were analyzed using HPLC, and the yield percentage was calculated by dividing sugar yield by maximum sugar yield possible. The errors presented were standard deviations of the duplicate experiments.

AFEX-Pretreated EPFBF at Various Conditions

To investigate the effectiveness of various AFEX conditions on the sugar yields, enzymatic hydrolysis at 1.0% glucan loading was conducted using Accellerase 1000 (15 FPU/g glucan; 84.4 μL/g dry EPFBF), Multifect Xylanase, and Multifect Pectinase (both at 24.7 μL/g dry EPFBF) for 24 h.

Optimization of Cellulase Loading

The level of cellulase (Accellerase 1000) required for maximum sugar yield was determined by varying cellulase enzyme loading from 0 to 35 FPU/g glucan (0–197 μL/g dry EPFBF) using a fixed hemicellulase mixture which consisted of 24.7 μL Multifect Xylanase and 24.7 μL Multifect Pectinase per gram of dry EPFBF. Enzymatic hydrolysis was conducted for 24 h.

Optimization of Hemicelluase Loading

Three commercial hemicellulolytic enzyme mixtures were tested in combination to formulate a mixture for high level sugar yield. These enzymes are Multifect Xylanese, Multifect Pectinase, and Research Enzyme NS series 50030 (RE 50030). A statistical tool (Simplex Design, Minitab) was used to design the combinations of the three enzymes to evaluate the importance of each for xylose yield. The pictorial illustration of the experimental design was as shown in Fig. 1. Every enzyme combination tested must contain hemicellulase enzymes at total protein concentration of 10 mg/g glucan. Enzymatic hydrolysis was conducted for 72 h. A contour plot was generated using Minitab statistical software using the monomeric xylose yield from the enzymatic hydrolysis. The protein concentration of Multifect Xylanese, Multifect Pectinase, and RE 50030 are 35, 90, and 16 mg/mL, respectively.

Accellerase and Multifect enzyme complexes were provided by Genencor (Palo Alto, CA, USA). RE50030 were provided by Novozymes North America (Franklinton, NC, USA).

Fermentation Using *Saccharomyces cerevisiae* 424A(LNH-ST)

Seed culture was prepared by inoculating from 424A(LNH-ST) frozen glycerol stock to a 100 mL YEPG solution (10 g/L yeast extract+5 g/L peptone+50 g/L glucose+50 mg/L

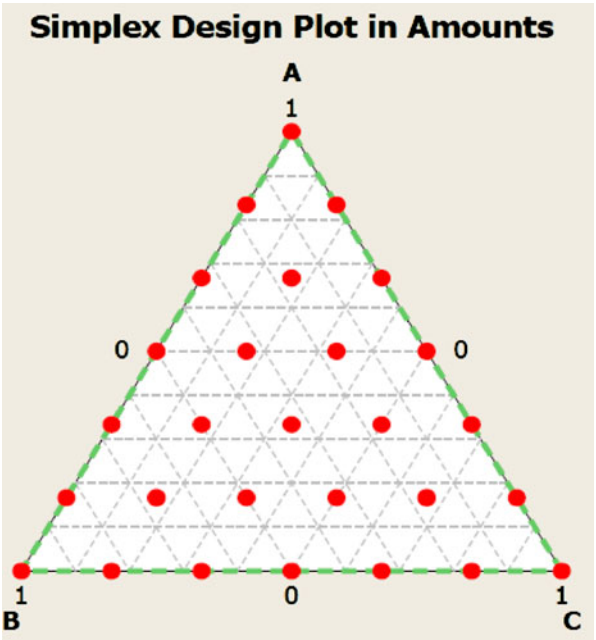

Fig. 1 Simplex statistical design for hemicellulolytic enzyme formulation. Note: (**a**), (**b**), and (**c**) represent each individual enzyme complex, 100%: 10 mg protein/g glucan

chloramphenicol) in a 250-mL unbaffled Erlenmeyer flask. The growth conditions for the seed culture were at 30 °C, 150 rpm agitation for 18–24 h. The grown seed culture was used to initiate fermentation.

Water extract at 3.0% glucan loading (approximately equal to 9.0% solids loading) was used to prepare the fermentation media. During the extraction step, water was preheated to 60–70 °C and a designated amount of AFEX-pretreated EBFPF was mixed with this water. The mixture was then pressed to obtain the water extract. This water extract was then again contacted with the previously washed material and pressed again for another two cycles. After completing the washing steps, the final water extract was used for fermentation. Other operational details on the extraction were as described in [12].

Prior to fermentation, the pH of the water extract was adjusted to 5.5. Glucose and xylose (reagent grade) were added to the water extract at a final concentration of 65 and 28 g/L, respectively. The seed culture for *S. cerevisiae* 424A(LNH-ST) was inoculated to an initial cell density of 2.0 (0.9 g dry wt/L). Fermentation was conducted at 30 °C, 150 rpm with a working volume of 70 mL in a 250-mL unbaffled Erlenmeyer flask under microaerobic conditions. Samples were taken at designated periods. The OD of the culture samples was measured at a wavelength of 600 nm. One unit absorbance at 600 nm is approximately equal to 0.45 g dry wt/L of the yeast cells.

Data Collection for Agricultural Residues from Oil Palm Plantation (Indonesia and Malaysia)

To estimate the potential of lignocellulosic residues from oil palm plantation, data on the oil palm harvested area and biomass generation per hectare per year were collected.

Palm plantation residues included in the calculation are felled palm trunk, palm frond, empty fruit bunch, palm mesocarp fiber, and palm kernel shell. The total biomass available for cellulosic ethanol production is estimated through Eq. 1

$$\text{Annual } \textit{total biomass}\,(\text{ton/year}) = (\text{total } \textit{palm harvested area}) \times \sum_0^i (\textit{type of annual biomass generation per hectare})i \quad (1)$$

i type of palm residues

Estimation of Maximum Fuel Production

The maximum capacity of cellulosic ethanol production was estimated by assuming 60% of the agricultural residues (regardless of the type of biomass) consisted of plant carbohydrate. Ethanol yield was assumed at 0.51 g ethanol per gram of monomeric sugar equivalent. The maximum potential of the cellulosic ethanol production from the agricultural residues was then calculated using Eq. 2.

$$\begin{aligned}&\text{Annual } \textit{cellulosic ethanol production potential}\,(\text{ton ethanol/year})\\ &= \textit{annual total biomass}(\text{ton/year}) \times \frac{0.6\ \textit{ton polymeric sugar}}{1.0\ \textit{ton biomass}}\\ &\times \frac{1.1\ \textit{ton monomeric sugar}}{1.0\ \textit{ton polymeric sugar}} \times \frac{0.51\ \textit{ton ethanol}}{1.0\ \textit{ton monomeric sugar}}\end{aligned} \quad (2)$$

Results

Parametric Exploration of AFEX Pretreatment on EPFBF

EPFBF used in this work contained 32.9% glucan, 22.4% xylan, and 1.4% arabinan. In this investigation, five batch AFEX treatments have been conducted to explore pretreatment conditions to overcome biomass recalcitrance. The effects of temperature, moisture content, and post-AFEX size reduction were evaluated by comparing the sugar yields in batch 1–3, batch 3 and 4, and batch 2 and 5, respectively (Fig. 2). Glucose yields after 24 h of enzymatic hydrolysis were about 40% in batch 2–4, where the tested pretreatment temperatures were between 135 and 155 °C. The difference in moisture contents on the tested pretreatment did not contribute to significant difference in sugar yields. Glucose yield increased by 52% when the overall particle size of the pretreated EPFBF was reduced post-AFE (to pass a 0.25-mm sieve). Compared to control results (0.25 mm ground untreated EPFBF), the glucose yield increased by 3-fold (Fig. 2); this increase is more dramatic for xylose yield (Fig. 2). However, both glucose and xylose yields displayed similar trends. Materials from batch 5 (see Table 1) were used for the following investigations.

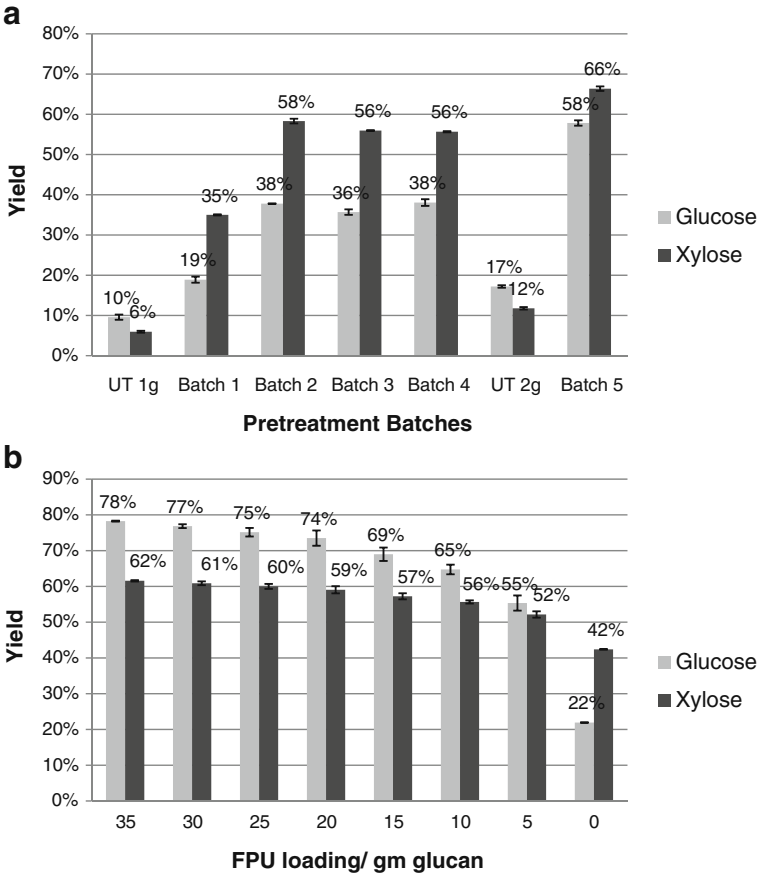

Fig. 2 **a** Monomeric glucose and xylose yield (24 h) for enzymatic hydrolysis of AFEX-pretreated EPFBF (pretreatment conditions as listed in Table 1). **b** Monomeric glucose and xylose yield (24 h) for enzymatic hydrolysis of AFEX-pretreated EPFBF on various concentrations of Accellerase (at fixed hemicellulase loading)

Sugar Yield vs Accellerase Loading

Enzymatic hydrolysis was conducted for 24 h on AFEX-pretreated EPFBF at a fixed loading of Multifect Xylanase and Multifect Pectinase across the tested range of Accellerase loading (0–35 FPU/g glucan). Glucose yield increased from 22% (no Accellerase) to 69% (at 15 FPU/g glucan loading) (Fig. 2b). The improvement margin on the higher loadings of Accellerase (up to 35 FPU/g glucan) is less than 9% (on glucose yield; Fig. 2b) and 5% (on xylose yield; Fig. 2b), relative to loading at 15 FPU/g glucan. An Accellerase loading of 15 FPU/g glucan was used to develop the hemicellulase mixture.

Development of the Hemicellulase Mixture

Three hemicellulase enzyme complexes, Multifect Xylanase, Multifect Pectinase, and RE 50030, were mixed at various ratios determined by a statistical tool (see Fig. 1).

The total hemicellulase loading was constrained at 10 mg protein/g glucan and the Accellerase loading was fixed at 15 FPU/g glucan. About 80% of the contour area indicated a xylose yield greater than 80% (Fig. 3a), suggesting that the existing hemicellulase complexes are sufficient to digest the hemicellulose in AFEX-pretreated EPFBF effectively. The contour area of the highest range of xylose yield (>95%) shifted downward (Fig. 3a), suggesting that Multifect Pectinase and RE 50030 were more effective complexes in hydrolyzing hemicellulase (in relative to Multifect Xylanase).

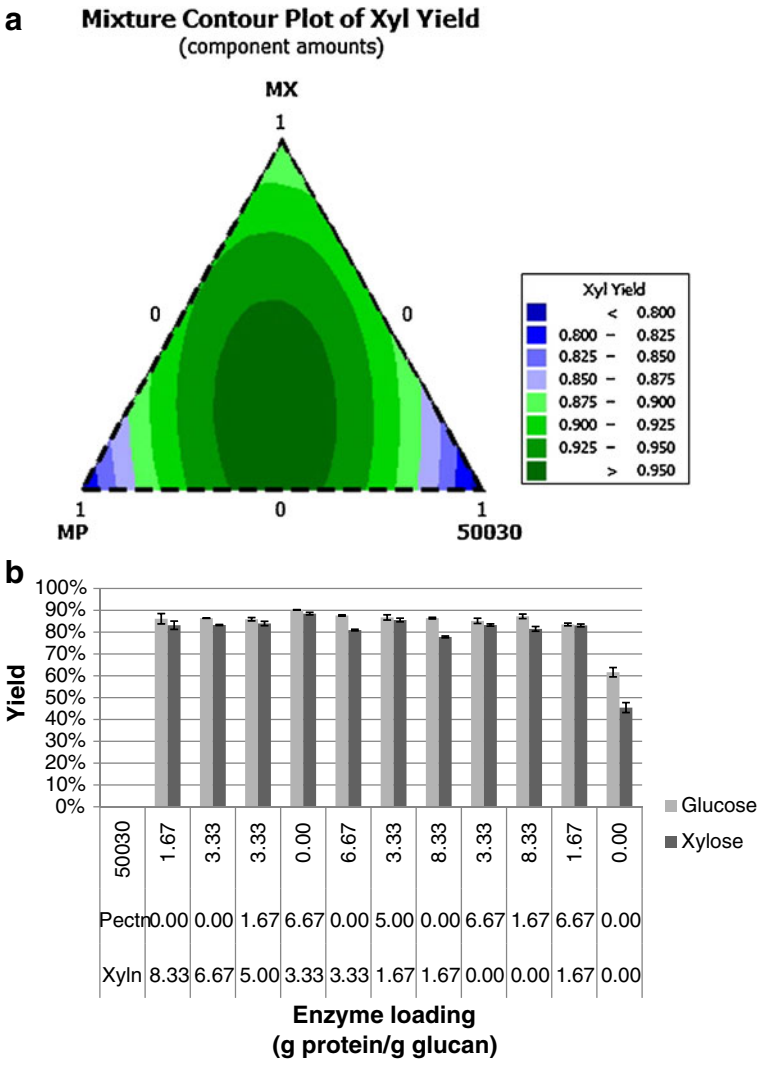

Fig. 3 **a** Evaluation of the effect of mixture ratio between Multifect Xylanase, Multifect Pectinase, and Research Enzyme 50030 on monomeric xylose yield using simplex statistical design plot. *MX* Multifect Xylanase, *MP* Multifect Pectinase, *50030* Research Enzyme #50030. Total hemicellulase loading constraint: 10.0 mg hemicellulase/g glucan. Accellerase loading was fixed at 15 FPU/g glucan. Enzymatic hydrolysis condition: pH 4.8, 50 °C, 200 rpm agitation. **b** Selected results for the monomeric glucose and xylose yield from development of hemicellulase mixture using simplex statistical design

However, the highest xylose yield among 32 tested enzyme combinations was achieved for the enzyme (protein weight) ratio between Multifect Xylanase/Multifect Pectinase/RE 50030 at 1:2:0 (Fig. 3b). The sugar concentrations at this enzyme loading yielded 10.1 g/L glucose and 6.8 g/L in the 1.0% glucan loading enzymatic hydrolysis.

Fermentation Using Water Extract from AFEX-Pretreated EPFBF

Water extract from the pretreated EPFBF contained nutrients and compounds generated by the pretreatment. Glucose fermentation using 9% solids loading equivalent of water extract was completed within 24 h. Xylose fermentation in the water extract was relatively slow (0.16 g/L/h; 0–72 h). Final ethanol concentration at 35.6 g/L was achieved at a metabolic yield of 91% (Fig. 4). Net cell growth during the fermentation was at 1.3 g dry wt/L (cell density at the end of fermentation was at 2.4 g dry wt/L).

Maximum Potential of Cellulosic Agricultural Residues from Palm Plantation in Malaysia and Indonesia

The maximum potential of palm residues for cellulosic ethanol production was estimated based on data from the year 2005. The total harvested area for both countries was almost equal at 3.6 million hectares. Close to 80% of the total residues on a dry weight basis is from palm fronds; this is followed by felled palm trunk, empty fruit bunches, mesocarp fiber, and kernel shell (Table 2). Assuming that the carbohydrate content of all of the palm residues is 60% of the total dry weight and they are effectively converted to ethanol at a yield of 0.51 g/g monomeric sugar, 49 billion liters of cellulosic ethanol can be produced each in both countries (Table 3). On an energy-equivalent basis, the maximum cellulosic ethanol potentials is 3.2- and 1.9-fold greater than the gasoline consumed Malaysia and Indonesia, respectively (Table 3).

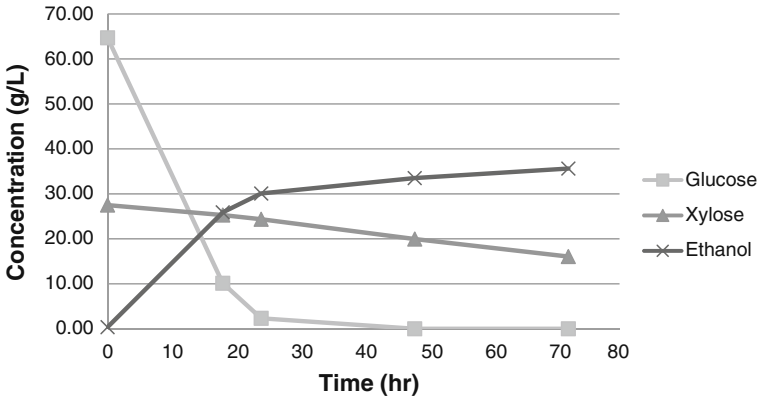

Fig. 4 Fermentation of water extract of AFEX-pretreated EPFBF with sugar addition using *S. cerevisiae* 424A(LNH-ST). Fermentation, initiated at 2.0 U of OD600 nm. Fermentation was conducted at pH 5.5, 30 °C, and 200 rpm agitation. Glucose (65 g/L) and xylose (28 g/L) were added to the water extract to provide a carbon source for fermentation. No other nutrients were added

Table 2 Cellulosic biomass available from palm plantation agricultural residues.

Type of biomass (cellulosic residues from palm plantation)	Dry ton/hectare/year[a]	Dry ton/year $\times 10^6$ (in Malaysia)[b]	Dry Ton/Year $\times 10^6$ (in Indonesia)[b]
Felled palm trunk	2.51	9.09	9.04
Palm fronds	24.87	90.03	89.53
Empty fruit bunches	1.55	5.61	5.58
Mesocarp fiber	1.63	5.90	5.87
Kernel shell	0.94	3.40	3.38
Total mass	31.50	114.03	113.40

Assumption: the values for dry ton/hectare/year for palm plantation in Malaysia and Indonesia are the same

[a] Source: Malaysia Palm Oil Council (MPOC, http://www.mpoc.org.my)

[b] Total palm plantation area in year 2005 ($\times 10^6$ ha): 3.62 (Malaysia), 3.60 (Indonesia); Source: Food and Agriculture Organization (FAO, http://faostat.fao.org). Calculations for dry ton/year are as described in Materials and Methods

Discussion

The Potential of Palm Agricultural Residues for Cellulosic Biofuels Production

The amount of cellulosic ethanol that could be produced from the existing cellulosic biomass from palm plantation (Table 2) from both Malaysia and Indonesia exceeds their gasoline consumption by 140% on an energy-equivalent basis (Table 3). The excess cellulosic ethanol could replace 63% of the gasoline consumption in China or 19% that of Europe based on 2005 consumption data [13].

The use of edible oil (such as palm oil) for biodiesel production has been a controversial issue due to its impacts on food prices and wildlife biodiversity due to deforestation in the producing countries [14]. However, by utilizing agricultural residues for cellulosic biofuels generation, we can increase the total revenue per hectare of palm plantation, replace fossil fuel, and potentially reduce the demand on the edible oil for liquid fuel production.

Table 3 Maximum capacity for ethanol production from palm residues and the scale of gasoline replacement.

		Malaysia	Indonesia
Total maximum cellulosic ethanol production[a]	$\times 10^6$ ton	38.38	38.17
	$\times 10^9$ L	48.65	48.38
Total maximum capacity for gasoline replacement ($\times 10^9$ L; after adjusting for the difference in energy content between gasoline and ethanol)[b]		32.11	31.93
Total motor gasoline consumption $\times 10^9$ L (2005)[c]		9.93	16.8

[a] Calculation method is as described in Materials and Methods

[b] Total maximum capacity for gasoline replacement (energy basis)=0.66×total maximum cellulosic ethanol production

[c] Source: International Energy Agency (IEA, http://data.iea.org/ieastore)

Why Bunch Fiber?

EPFBF could be the first cellulosic feedstock from palm residues because whole fresh palm fruit bunches, under the existing practice, are collected and transported to centralized oil palm processing facilities. Therefore, the transportation logistics for this biomass have been established. The collected EPFBF could be further processed for cellulosic biofuels production on a commercially relevant scale. In contrast, palm fronds, although they are the most abundant palm residues, are not collected or processed under existing practices.

AFEX, Enzymatic Hydrolysis, and Fermentability of AFEX-Pretreated EPFBF

AFEX pretreatment condition, enzymatic hydrolysis, and the fermentability of the pretreated EPFBF were investigated. Using the enzyme formulation developed (within the specified formulation constraints), close to 90% of the sugars (glucose and xylose) are digested to monomers. The total sugar yields from the enzymatic hydrolysis of AFEX-pretreated materials at solids loading that ranged from 1% to 6% glucan loading are shown to be comparable [7, 15, 16].

Thus, the biomass processing strategy is also likely to be effective to convert plant carbohydrate to fermentable sugars at high concentration. However, post-AFEX size reduction is required to enhance the sugar yield, a processing step that is not reported in the works on other AFEX-pretreated cellulosic biomass [8, 15]. One explanation of this phenomenon is the high tensile strength (248 MPa) and toughness (2,000 MPa) of palm fiber compared to most cellulosic feedstocks [17].

Fermentation of the water extract from AFEX-pretreated EPFBF at 9% solids loading confirms the fermentability of this pretreated material. The dissolved nutrients from the pretreated biomass can support yeast growth to ferment glucose up to 65 g/L within 24 h, in agreement with previous reports on the fermentability of AFEX-pretreated substrates [7, 9, 18].

From these results, we can conclude that 90% of the plant carbohydrate can be converted to monomeric sugars with a highly fermentable plant hydrolysate without requiring further nutrient supplementation during fermentation or detoxification to remove inhibitory substances.

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