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THE IMPROVEMENT ON ENZYMATIC HYDROLYSIS OF OIL PALM (*Elaeis guineensis*) EMPTY FRUIT BUNCH LIGNOCELLULOSE

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ABSTRACT

The lignocellulose constituents of oil palm biomass have potential as substrates for sugar and bioethanol production. Research focusing on this indicated that high enzyme loading may hinder large-scale utilisation. Thus, attempts improving the enzymatic saccharification of the acid-pretreated empty fruit bunches (EFB) were made by investigating enzyme (cellulase) concentrations and combinations, and co-existence of enzyme with catalysing agents e.g. metal ions and surfactants. The study revealed that a combined Celluclast 1.5 L: Cellic CTec of 30 FPU/g: 1% (v/v) gave the optimum glucose yield of 307.3 ± 3.5 mg/g. The presence of 10 mM cobalt ion and 0.5% (v/v) Triton X-100 enhanced cellulose conversion by 16.2% and 38.4%, respectively. Overall, an addition of non-ionic surfactants gave the most satisfied cellulose conversion (83.8%) corresponding to 567.2 ± 28.4 mg/g of glucose yield from the pretreated EFB. This improved enzymatic saccharification can be utilised to enhance the production of second generation bioethanol, particularly from oil palm biomass.

Key words: Oil palm empty fruit bunch, Enzymatic saccharification, Cellulose conversion, Fermentable sugar, Bioethanol

INTRODUCTION

Extensive studies have geared towards developing bioethanol from various types of lignocellulosic biomass due to concerns for the environment and energy security. Lignocellulosic biomass is renewable, biodegradable and available in abundance. One such major biomass available in Malaysia is oil palm empty fruit bunches (EFB) which is a by-product from the palm oil milling process. An annual production of EFB amounting to ~7.7 million tonnes (dry basis) a year from nearly 440 palm oil mills over 5.23 million ha of oil palm planted area (MPOB, 2013) implies that EFB is available abundantly, and replenishable, hence is a potential feedstock to provide intermediate platforms for the production of bioenergy and biochemicals. E.g. C5 and C6 from EFB can become building blocks for bioethanol production; an alternative fuel to gasoline.

EFB consists primarily of cellulose, hemicellulose, lignin and ash. These constituents are cross-linked and associated with each other via various different bonding, hence difficult to be broken apart. The cellulose being a major composition of EFB, is a glucose-based polymer which can potentially be recovered and converted into bioethanol and other value-added products, if it is exposed and made more accessible to cellulase enzymes (Cui *et al*., 2014). Generally, bioethanol from EFB can be produced via pretreatment of lignocellulose to separate the lignin, hydrolysis/ saccharification of cellulose to release the simple fermentable sugars and fermentation of sugars to bioethanol followed by product purification. Enzymatic hydrolysis of lignocellulosic biomass has been identified as a "green" and environmentally-friendly process (Osman *et al.,* 2013). However, many factors, e.g. lignin content, cellulose crystallinity, polymerization degree, moisture content and available surface area could limit the digestibility of the hemicellulose and cellulose present in the lignocellulosic biomass by enzyme, hence, the main constraints in bioconversion (Cui *et al*., 2014; Hendriks and Zeeman, 2009; Mood *et al.,* 2013). To address this,

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proper pretreatment and enzyme requirement in hydrolysis prior to fermentation must be evaluated. In this study, effects of various cellulases concentrations and the presence of metal ions and surfactants on EFB hydrolysis were studied. The conversion efficiency of the pretreated samples was evaluated based on glucose recovery in respect of the cellulose present in the biomass.

MATERIALS AND METHODS

Raw materials

EFB fibres were collected from a palm oil mill in Klang, Malaysia. The fibres were oven dried at 100 ± 5°C and cut into smaller pieces. They were milled, sieved and separated into fractions of different sizes using a test sieve shaker (EFL2000, Endecotts, England). The size of the EFB used for this study was standardized around 91-106 μm.

Pretreatment of EFB

The dried EFB at 10% (w/v) solid loading were mixed with dilute sulphuric acid solution (1% v/v). The mixture was then autoclaved (HICLAVE HVE-50, Hirayama, Japan) for 90 mins at 125°C. The pretreated EFB fibres were washed thoroughly with hot distilled water to a neutral pH and oven dried at 80 ± 5°C prior to enzymatic reaction.

Enzymatic saccharification of EFB

The pretreated EFB (5% w/v) were suspended in 50 mM citrate buffer (pH 4.8) and added with cellulase having activity of 7.5-45.0 FPU/g dry pretreated solid (filter paper unit, FPU). A combination of commercial enzymes i.e. Celluclast 1.5 L, Cellic CTec and Cellic HTec (Novozymes A/S, Bagsvaerd, Denmark) at different concentrations were performed to find the best enzyme loading for saccharification. In addition, the presence of metal ions - copper, iron, zinc, manganese, magnesium, nickel, cobalt and calcium - and surfactants - Tween 20 (polyoxyethylene sorbitan monolaurate), Tween 80 (polyoxyethylene sorbitan monoleate) and Triton X-100 ($C_{34}H_{62}O_{11}$) - in the saccharification process was conducted to find the optimum yield of glucose. The metal ions and surfactants at different molarities (5 mM and 10 mM) and different percentages (0.5%, 1.0%, 2.0% and 3.0%, v/v), respectively were also performed. The samples were incubated at an optimum conditions (50°C, 150 rpm for 72 h) established previously (Mohd Asyraf *et al.,* 2011). Sample aliquots were withdrawn at every 24-h interval and analysed for the released sugars. Equation 1 was used to calculate the conversion of glucose from cellulose after enzymatic saccharification process.

$$\text{Celulose conversion (\%)} = \left[\frac{\text{Amount of glucose released}}{\text{Amount of cellulose in the pretreated EFB}}\right] \times 100 \quad \text{(Eq. 1)}$$

Analytical methods

The compositional analyses of the raw and pretreated EFB samples were conducted according to the standard methods: TAPPI T 222 om-11 to extract lignin; ASTM D 1104-56 to extract holocellulose; ASTM D 1103-60 to remove hemicellulose fraction from the holocellulose and ash content by heating the sample to 750°C for 120 min using Thermo Gravimetric Analyzer (TGA710, LECO, USA).

Sugar contents of the hydrolysed samples were determined using high performance liquid chromatography (HPLC) (Waters 2707, USA) equipped with refractive index detector (Waters 2414, USA using deionized water as the mobile phase; Sugar Pack™ column: 6.5 x 300 mm, column temperature: 75°C; flow rate: 0.5 ml/min and injector volume of 10 μL. All samples were filtered through 0.45 μm PTFE membrane filters and injected to the column. The data was acquired and analysed using Breeze software (Waters, USA).

RESULTS AND DISCUSSION

Compositional analysis

The raw and pretreated EFB fibres were characterised (Table 1). The raw EFB consisted of 73.6% holocellulose (43.5% cellulose and 30.0% hemicellulose), 23.5% lignin and 3% ash. After dilute acid pretreatment, the cellulose content increased to 67.7% while hemicellulose, lignin and ash decreased to 17.6% 14.3% and 0.45%, respectively. The results showed that the employed dilute acid pretreatment (1% v/v) had significantly removed lignin (39% removal) and improved the cellulose content (55.6%) required for the subsequent enzymatic saccharification to enhance the release of glucose.

Effect of enzyme concentrations and combinations on saccharafication of EFB

Cellulase is commonly used to hydrolyze lignocellulosic biomass in order to depolymerize cellulose to glucose. Cellulases are categorised in three major classes i.e. endoglucanases, exoglucanases and α-glucosidases (Ghosh and Ghose, 2003). In this study, enzymatic saccharification of the dilute acid pretreated EFB using a combination of enzymes (Celluclast 1.5 L, Cellic CTec and Cellic HTec, B1-B7) vs. single enzyme (Celluclast 1.5 L) at different concentrations (A1-A6) was carried out for 72 h. During the course of this process, a regular increase in glucose yield

Table 1. Percentage improvement of the chemical compositions of empty fruit bunch fibre before and after pretreatment

Samples	Composition (dry weight, %)				
	Holocellulose	α-cellulose	Hemicellulose	Lignin	Ash
Raw EFB	73.57 ± 1.4	43.48 ± 1.2	30.09 ± 0.2	23.45 ± 1.5	2.98 ± 0.1
Pretreated EFB	85.25 ± 1.6	67.67 ± 1.9	17.58 ± 2.3	14.30 ± 0.2	0.45 ± 0.1
Percentage improvement	15.97	55.63	41.58	39.02	84.90

Table 2. Glucose yield and cellulose conversion efficiency at 24, 48 and 72 h of enzymatic hydrolysis using different enzyme loadings

Sample	Enzyme loadings			Glucose yield (mg/g pretreated EFB*)			Cellulose conversion (%)		
	Celluclast 1.5L (FPU/g)	CTec (%)	HTec (%)	24 h	48 h	72 h	24 h	48 h	72 h
A1	7.5	–	–	55.3 ± 4.6	74.0 ± 2.3	89.5 ± 7.7	8.12	10.93	13.29
A2	15.0	–	–	84.9 ± 6.2	115.0 ± 6.3	145.0 ± 10.4	12.56	16.99	21.42
A3	22.5	–	–	91.8 ± 7.6	124.5 ± 4.2	151.7 ± 12.0	13.59	18.46	22.45
A4	30.0	–	–	106.0 ± 10.3	146.0 ± 3.5	175.9 ± 5.4	15.66	21.57	26.00
A5	37.5	–	–	107.3 ± 5.8	135.0 ± 1.6	157.6 ± 1.3	15.81	19.94	23.34
A6	45.0	–	–	111.4 ± 9.5	148.5 ± 11.4	188.9 ± 9.9	16.40	22.01	27.92
B1	15.0	1.00	1.00	152.0 ± 13.7	196.5 ± 11.5	238.6 ± 19.6	22.45	29.10	35.30
B2	22.5	1.50	–	154.6 ± 12.7	202.5 ± 13.4	251.1 ± 19.1	22.90	29.99	37.08
B3	22.5	1.00	0.50	174.3 ± 14.9	223.5 ± 12.7	270.0 ± 18.7	25.70	33.09	39.88
B4	30.0	0.50	0.50	181.1 ± 10.9	239.5 ± 12.0	300.3 ± 3.5	26.74	35.45	44.31
B5	30.0	1.00	–	165.7 ± 13.6	234.5 ± 16.3	307.2 ± 0.5	24.52	34.71	45.35
B6	37.5	0.50	–	179.5 ± 12.2	237.0 ± 14.4	297.6 ± 5.4	26.59	35.01	44.02
B7	37.5	0.45	0.05	177.0 ± 14.9	239.5 ± 17.2	305.3 ± 1.1	26.14	35.45	45.05

* Cellulose content of pretreated empty fruit bunches (EFB): 67.7%

was observed throughout the reaction time in all samples (Table 2). In control samples A, the conversion efficiency increased with increasing of Celluclast 1.5 L concentration ranging from 13.3-27.9% with an enzyme concentration of 7.5-45 FPU/g at 72 h. Samples B, on the other hand, showed overall improved efficiency and achieved a maximum cellulose to glucose conversion (45.4%) in the combined Celluclast 1.5 L and Cellic CTec sample, B5. This finding showed that the presence of Cellic CTec in Celluclast 1.5 L improved the glucose yields, while the co-existence of Cellic HTec brought about just a small change, compared to the control reactions containing only Celluclast. Thus, the optimised cellulase combination i.e. 30 FPU/g supplemented with 1.0% (v/v) Cellic CTec was used in the following experiment.

Effect of metal ions on enzymatic saccharafication of EFB

Metal ions may influence the catalytic activity of enzyme by acting as enzyme cofactors or inhibitors (Schiffmann *et al.,* 2005) dependent on the charges exhibited. The effect of metal ions (divalent cations) on enzymatic hydrolysis is shown in Fig. 1 and Table 3. The resulting glucose yields were compared relatively to the control set at 100% in Fig. 1. The addition of metal ions caused an overall increase in glucose yields at 5 mM and 10 mM concentration, respectively except for Cu^{2+} at 10 mM showing otherwise. This reduction could be due to the nature of the metal ion that inhibits the working enzymes. The reduced glucose production can be due to denaturation of the active sites of the enzymes. Co^{2+} was the most stimulating with 49.1% enhancement in relative glucose yield at 10 mM (Fig. 1), i.e. an increment of glucose yield from 222.4 ± 4.6 mg/g to 331.6 ± 5.7 mg/g of pretreated EFB (Table 3). This finding was correponded well with previous studies which reported that Co^{2+} and Mn^{2+} could increase cellulase activity from *Bacillus subtilis* (Yin *et al.,* 2010), *Mucor circinelloides* (Saha, 2004) and *Chalara paradoxa* (Lucas *et al.,* 2001).

Effect of surfactants on enzymatic hydrolysis of EFB

In general, hydrolyses of cellulose to glucose by cellulase enzyme suffers from slow reaction rates

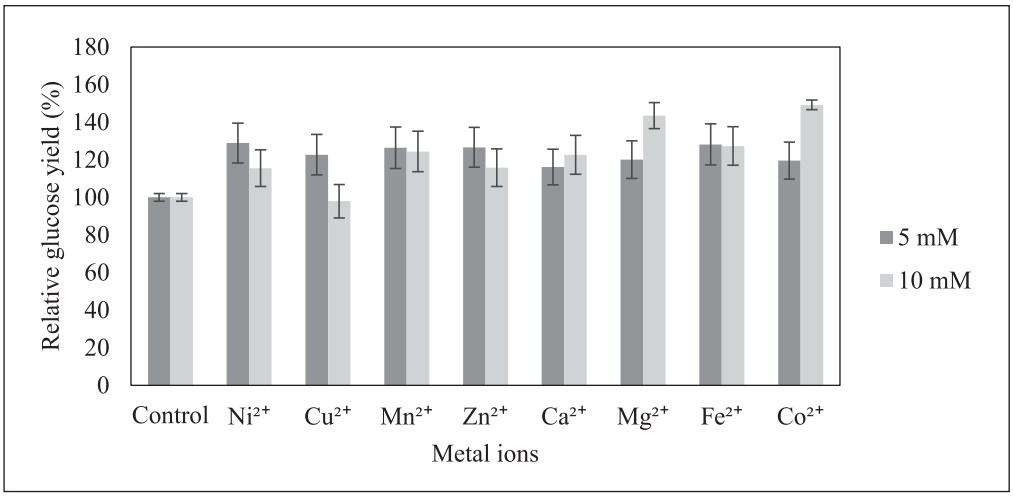

Fig. 1. Effect of metal ions on cellulase activity as measured by glucose yield during 72 h of enzymatic saccharification.

Table 3. Amount of glucose yield and cellulose conversion efficiency at 24, 48 and 72 h of enzymatic hydrolysis with different metal ions

Metal ions	Glucose yield (mg/g pretreated EFB)						Cellulose conversion (%)					
	5 mM			10 mM			5 mM			10 mM		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Ctrl*	176.8 ± 19.7	216.6 ± 7.4	222.4 ± 4.6	176.8 ± 19.7	216.6 ± 7.4	222.4 ± 4.6	26.1	32.1	32.8	26.1	32.1	32.8
Ni^{2+}	178.8 ± 21.1	243.8 ± 26.5	286.0 ± 23.6	168.0 ± 19.7	226.2 ± 24.2	256.8 ± 21.7	26.4	36.0	42.2	24.8	33.4	38.0
Cu^{2+}	200.8 ± 22.3	244.2 ± 25.8	272.2 ± 23.9	169.0 ± 18.8	206.8 ± 21.2	217.8 ± 19.8	29.7	36.0	40.2	25.0	30.6	32.2
Mn^{2+}	199.4 ± 22.6	252.8 ± 26.7	280.4 ± 24.4	195.2 ± 22.3	250.8 ± 26.4	276.6 ± 24.1	29.4	37.4	41.4	28.8	37.1	40.9
Zn^{2+}	183.4 ± 21.3	242.8 ± 26.2	281.0 ± 23.6	172.0 ± 20.3	233.6 ± 24.6	257.4 ± 22.1	27.0	35.9	41.5	25.4	34.6	38.0
Ca^{2+}	157.0 ± 18.5	213.8 ± 23.6	257.8 ± 21.0	176.8 ± 21.0	243.8 ± 25.8	272.6 ± 23.1	23.3	31.6	38.1	26.1	36.0	40.3
Mg^{2+}	175.8 ± 19.8	220.8 ± 24.4	266.6 ± 22.1	174.8 ± 21.1	246.2 ± 28.3	319.0 ± 15.4	26.0	32.6	39.4	25.8	36.3	47.1
Fe^{2+}	196.8 ± 22.2	248.0 ± 26.6	284.4 ± 24.3	169.2 ± 19.8	227.4 ± 25.5	283.0 ± 22.7	29.1	36.6	41.9	25.0	33.5	41.8
Co^{2+}	164.2 ± 19.4	223.6 ± 24.5	265.4 ± 21.8	180.8 ± 21.9	257.2 ± 9.4	331.6 ± 5.7	24.2	33.1	39.1	26.7	38.0	49.0

* Ctrl = Control contains only enzyme: 30 FPU/g + 1% (v/v) Cellic CTec, without metal ion addition

due to highly crystalline structure of cellulose. This makes the penetration of enzymes to the active sites very difficult (Kumar *et al.,* 2009). Addition of surface acting agents (surfactants) during enzymatic saccharification is capable of modifying the surface property of cellulose and minimizing the irreversible binding of cellulase on cellulose (Sun and Cheng, 2002). Surfactants were used in the saccharification process as a carrier enzyme during enzyme-substrate interaction (Rashid *et al.,* 2011). Alkasrawi *et al.* (2003) showed that the addition of surfactants was able to improve the enzymatic hydrolysis of several varieties of cellulose-containing substrates. Kurakake *et al.* (1994) proved that non-ionic surfactants could enhance cellulose hydrolysis while their anionic and cationic are proven counterparts decreased the cellulose activity in hydrolysis. Therefore, non-ionic surfactants were selected and applied in this study. Fig. 2 and Table 4 show the effect of surfactants on enzymatic saccharification of EFB for 72 h reaction time. The longer the saccharification time, the better in glucose yield throughout the saccharification period (Table 4). It was found that supplementation of surfactants (Triton X-100, Tween 20 and Tween 80) at lower concentrations gave higher glucose yields with 85.0%, 67.9% and 41.7% enhancement in relative glucose yield at 0.5% (v/v) each compared to the unsupplemented control (Fig. 2). Overall, Triton X-100 gave the highest glucose yield and cellulose conversion efficiency (83.8%) among the surfactants used after 72 h of enzymatic saccharification of EFB (Table 4).

CONCLUSION

The factors affecting the enzymatic saccharification of cellulose of EFB in this study were the types of enzymes loading (Cellic CTec and Cellic HTec) and the co-existence of metal ions and non-ionic surfactants in performing cellulase reactivity. It was shown that CTec, Co^{2+} and Triton X-100 outperformed that of HTec, other covalent metal ions

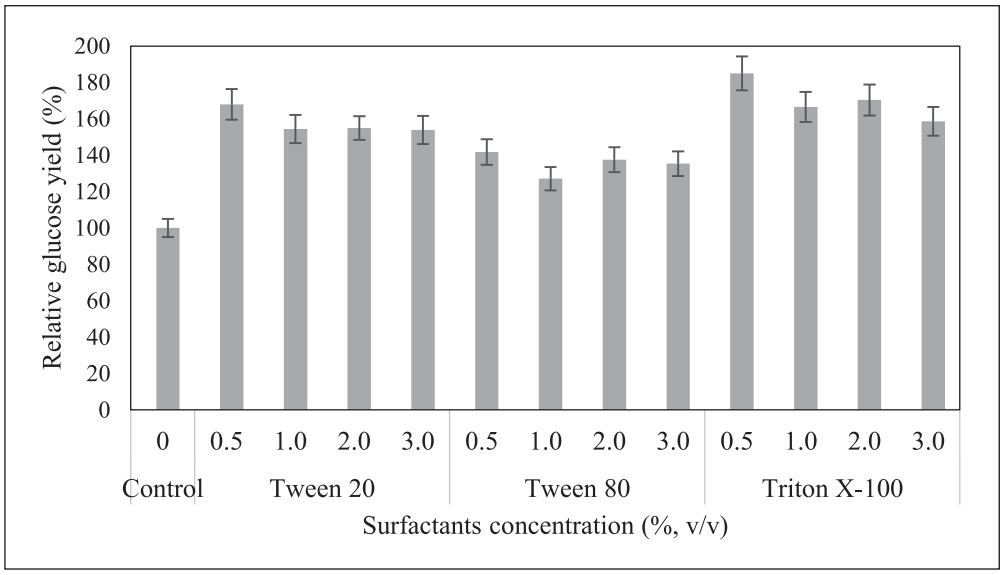

Fig. 2. Effect of surfactants on cellulase activity as measured by glucose yield during 72 h of enzymatic saccharification.

Table 4. Amount of glucose yield and cellulose conversion efficiency at 24, 48 and 72 h of enzymatic hydrolysis with different types and concentrations of surfactants

Surfactant	Concentration (%, v/v)	Glucose yield (mg/g pretreated EFB)			Cellulose conversion (%)		
		24 h	48 h	72 h	24 h	48 h	72 h
	Control*	237.6 ± 11.8	306.6 ± 15.3	306.6 ± 15.3	35.16	45.35	45.35
Tween 20	0.5	325.6 ± 16.3	445.6 ± 22.3	514.8 ± 25.7	48.15	65.88	76.07
	1.0	341.8 ± 17.1	439.2 ± 22.0	473.4 ± 23.7	50.52	64.84	69.87
	2.0	365.4 ± 20.0	454.0 ± 20.0	475.0 ± 20.0	53.91	67.06	70.16
	3.0	376.8 ± 18.8	477.0 ± 23.9	471.8 ± 23.6	55.69	70.46	69.72
Tween 80	0.5	273.0 ± 13.7	375.4 ± 18.8	434.6 ± 21.7	40.32	55.39	64.25
	1.0	312.2 ± 15.6	363.6 ± 18.2	389.6 ± 19.5	46.09	53.77	57.61
	2.0	310.8 ± 15.5	407.8 ± 20.4	421.8 ± 21.1	45.94	60.27	62.33
	3.0	314.0 ± 15.7	414.8 ± 20.7	415.0 ± 20.8	46.38	61.30	61.30
Triton X-100	0.5	394.2 ± 19.7	517.0 ± 25.9	567.2 ± 28.4	58.20	76.37	83.75
	1.0	385.6 ± 19.3	482.0 ± 24.1	510.6 ± 25.5	57.02	71.20	75.48
	2.0	409.2 ± 20.5	521.2 ± 26.1	522.2 ± 26.1	60.41	76.96	77.10
	3.0	404.6 ± 20.2	507.0 ± 25.4	486.2 ± 24.3	59.82	74.89	71.79

* Contains only enzyme: 30 FPU/g + 1% (v/v) Cellic CTec, without surfactant addition

and non-ionic surfactants, in enhancing the activity of Celluclast 1.5L. This led to the highest glucose yield of 567.2 ± 28.4 mg/g and cellulose conversion efficiency of 83.8% from pretreated EFB having a cellulose content of 67.7%.

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