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Bioethanol Production by Fermentation of Oil Palm Empty Fruit Bunches Pretreated with Combined Chemicals

Nurul Adela, B.*, Nasrin, A.B., Loh, S.K. and Choo, Y.M.

Energy and Environment Unit, Engineering and Processing Research Division, Malaysian Palm Oil Board (MPOB), 6, Persiaran Institusi, Bandar Baru Bangi, 43000 Kajang, Selangor, Malaysia.

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ABSTRACT

: Oil palm empty fruit bunches (EFB) contains about 73.6% (w/w) holocellulose and can serve as a renewable feedstock for bioethanol production. This study investigated the bioethanol production from chemically-pretreated EFB via enzymatic saccharification and fermentation. EFB was pretreated with 1.0% (v/v) dilute H_2SO_4 at 125°C for 90 min followed by 1% (w/v) NaOH at 100°C for 60 min. The combined chemical pretreatment was able to remove >90% of the hemicellulose and 50% of lignin. The delignified EFB (5.0%, w/v) containing mostly cellulose was enzymatically hydrolysed for 72 h to yield 484.79 ± 0.65 mg/g of glucose. Furthermore, the addition of non-ionic surfactant i.e. 0.5% (v/v) Triton X-100 enhanced saccharification by 31.3%. The SEM analysis revealed that, the combined chemicals changed the EFB morphology by removing the chemical compositional barrier i.e. silica and altering the physical structural impediment by formation of pores after silica removal, thus providing more surface areas for enzymatic attack. The EFB-derived sugar was fermented by *Saccharomyces cerevisiae* to produce 12.13 ± 0.99 g/L of bioethanol with theoretical yield of 89.1% within 24 h. The findings value add to the current pretreatment of lignocellulosic biomass particularly for bioethanol production and other renewable resources.

KEYWORDS: Oil palm empty fruit bunches, Pretreatment, Fermentable sugar, Enzymatic saccharification, Bioethanol

INTRODUCTION

Extensive studies attempting to develop bioethanol from various types of lignocellulosic biomass have been carried out because they are renewable and abundant. An example of such a major lignocellulosic biomass available in Malaysia is empty fruit bunches (EFB) which are generated during the palm oil milling process. In 2012, the annual production of EFB was estimated to be 7.4 million tonnes from nearly 440 palm oil mills over an oil palm planted area of 5.08 million ha [1]. This implies that EFB is available abundantly and is likely to be a potential intermediate for the production of liquid fuel i.e. bioethanol; an alternative fuel to gasoline. In fact, bioethanol can be an oxygenated fuel to increase its oxygen content, causing better hydrocarbon oxidation and mitigating greenhouse gases than gasoline [2, 3].

Generally, bioethanol from EFB can be produced via pretreatment of lignocellulose, hydrolysis/ saccharification of cellulose to produce simple fermentable sugars, fermentation of sugars to bioethanol and product separation. EFB consists primarily of cellulose, hemicellulose, lignin, and ash which are associated with each other. Many factors, e.g. lignin content, cellulose crystallinity, polymerization degree, moisture content and available surface area limit the digestibility of the hemicellulose and cellulose present in the lignocellulosic biomass, hence, the main constraint in bioconversion [4, 5]. To address this, proper pretreatment and delignification process is necessary; enabling the breaking down of the complex structures of lignocelluloses, reducing the lignin and hemicellulose contents vis-á-vis an increase in the pore size and surface area, thus increasing the accessibility of cellulose for enzymatic digestion [6, 7, 8, 9]. In view of this, an effective pretreatment process of EFB is undeniably very crucial if a highly hydrolysed sugars yield for the overall improvement of bioethanol were to be pursued. Many studies in this area were conducted, but far none made satisfactory progress on pretreatment.

In this study, we developed a pretreatment method for subsequent bioethanol production from EFB. A combined chemical pretreatments - a dilute acid to remove hemicellulose and a few delignifying agents to remove lignin - was attempted to enhance the cellulose proportion in the EFB prior to enzymatic hydrolysis. The recovered hexose sugars after enzymatic hydrolysis was subsequently fermented using selected yeast strain to produce bioethanol. The morphological and structural changes of EFB during each treatment were examined using scanning electron microscope (SEM) to provide a better understanding on this improved bioconversion method.

***Corresponding Author:** Nurul Adela B., Engineering and Processing Research Division, Malaysian Palm Oil Board (MPOB), 6, Persiaran Institusi, Bandar Baru Bangi, 43000 Kajang, Selangor, Malaysia.
E-mail: adela@mpob.gov.my Tel.: +603 87694264; fax: +603 89263827

MATERIALS AND METHODS

EFB collection and preparation

EFB was collected from a local palm oil processing mill in Klang, Malaysia. The bunch was dried at 100 ± 5°C and cut into smaller pieces. It was then milled, sieved, and separated in fractions using a test sieve shaker (Endecotts EFL 2000). The particle size of EFB used for this study was in the range of 91-106 μm.

Bioconversion of EFB

1. Chemical pretreatment of EFB

The dried EFB at 10% (w/v) solid loading (10 g EFB in 100 ml H_2SO_4) were hydrolysed in H_2SO_4 solution at different concentrations. The process parameters to be examined and optimised were: temperature (105 and 125°C), holding time (90 and 120 min) and acid concentration (0.5–3.0%, v/v). The hydrolysed biomass was washed with hot water to a neutral pH. The hydrolysate was analysed for the content of sugars, and the solid biomass was dried overnight at 80°C prior to delignification.

The optimised acid-pretreated EFB residue was subsequently delignified with different delignifying agents i.e. NaOH, $NaClO_2$, and H_2O_2 at 2.0% (w/v) concentration at 100°C for 1 h. The delignified biomass was then filtered and the cellulosic residue was washed thoroughly with hot water and dried overnight at 80°C.

2. Enzymatic saccharification of pretreated EFB

The enzymatic hydrolysis was performed using a commercial cellulase derived from *Trichoderma reesei* (Novozymes A/S, Bagsvaerd, Denmark). The amount of enzyme used was 30 FPU U/g of dry pretreated solid (filter paper unit, FPU). One unit of FPU is defined as the enzyme amount which converts 1 μmol glucose equivalents from Whatman no. 1 filter paper in 1 min reaction time [10]. Three non-ionic surfactants were tested i.e. Tween 20, Tween 80 and Triton X-100 at 0.5% (v/v) concentrations to enhance saccharification and to compare with the NaOH-pretreated EFB which is surfactant-free. The solid loading of 5.0% (w/v) EFB were suspended in 50 mM citrate buffer (pH 4.8) and added with cellulase together with respective surfactants. The samples were incubated at 50°C, 150 rpm for 72 h. Sample aliquots were withdrawn at 24 h interval and analysed for the released sugars.

3. Microorganism growth conditions and fermentation

Saccharomyces cerevisiae ATCC 24860 was grown on YPD agar (1% yeast extract, 2% peptone, 2% glucose and 1% agar) at room temperature for 3 days. To prepare a seed culture for bioethanol fermentation, *S. cerevisiae* was cultivated in 10 ml YPD medium (1% yeast extract, 2% peptone and 2% glucose) for 18 h. Log phase cultures of *S. cerevisiae* (10%, v/v) were used as inoculum and inoculated into a saccharified medium. The samples were fermented employing optimised conditions as described in previous work [11] i.e. pH 4.0, temperature of 30°C and agitation of 150 rpm. Sample aliquots were withdrawn at every 24 h interval up to 72 h and quantified for its sugars consumption and bioethanol formation. Cell density was measured via absorbance at 600 nm using UV/Vis spectrophotometer (Genesys 20, Thermo Scientific, USA).

Analytical procedures

1. Lignocellulosic compositions

The chemical compositions of pulverised EFB was analysed according to ASTM 1104-56 and ASTM D1103-60 method for holocellulose and α-cellulose, respectively. For the gravimetric method of lignin determination, 0.5 g of sample was weighed into a 100-ml Erlenmeyer flask and stirred for 2 h in 10 ml of cold 72% (v/v) H_2SO_4 solution. The mixture was transferred into a 500-ml beaker and boiled for 4 h in 300 ml distilled water under continuous stirring. The mixture was filtered off using glass microfiber filter grade GF/B (Whatman) in porcelain crucible. The residue retained on the filter was washed with hot water until it was acid-free and allowed to dry at 105°C for 2 h and weighed.

The holocellulose - a composite of cellulose and hemicellulose - was extracted from EFB using acidified sodium chlorite method. Approximately 24.0 g of sample was mixed with 960 ml distilled water and treated with 3.0 ml acetic acid and 9.0 g sodium chlorite at 70-80°C for 4 h under continuous stirring. The mixture was then washed with hot water, filtered and dried at 105°C for 24 h. Determination of holocellulose was carried out using dry weight method. A total of 12.0 g of dried holocellulose obtained was further dissolved in 240 ml of 17.5% (v/v) NaOH solution and stirred for 30 min. A total of 60 ml of NaOH solution was added into the mixture and allowed to mix to separate hemicellulose from the holocellulose and leaving α-cellulose. The insoluble α-cellulose was filtered and washed separately with 8.3% (v/v) NaOH solution followed by 10% (v/v) acetic acid. The α-cellulose was finally washed with hot water to a neutral pH and dried overnight at 80°C.

The ash content (% on dry basis) were determined by heating the sample from room temperature to 750°C for 120 min using thermogravimetric analyzer (TGA) (TGA710, LECO, USA).

2. Scanning electron microscopic (SEM)

The acid and alkali pretreated biomass samples were subjected to microscopic study. The residue remaining after enzymatic saccharification was also selected for microscopic observation. The sample from each pretreatment was filtered and washed using hot water prior to drying at 80°C for 24 h. The dried sample was subjected to SEM using a Hitachi S-3400N model with Energy-dispersive X-ray spectroscopy (Horiba EMAX EDX) (SEM-EDX) under backscattered electrons (BSE) mode with 15 kV.

3. Products quantification

Sugars and ethanol concentrations were analysed using high performance liquid chromatography (HPLC) (Waters 2707). Sugar Pack™1 column was used for the analysis. Samples were filtered through 0.45 µm PTFE membrane filters and injected to the column. The deionized water was used as the mobile phase with flow rate of 0.5 ml min^{-1}. The column temperature was maintained at 75°C.

The ethanol yield (g g^{-1}) was calculated based on experimental ethanol produced and expressed as g ethanol per total g of sugar utilised (Eq. 1) and the fermentation efficiency or theoretical ethanol yield (%) was calculated based on the ratio of ethanol yield obtained against theoretical maximum ethanol yield (Eq. 2). The ethanol productivity was calculated based on ethanol produced against fermentation time (Eq. 3)

$$\text{Ethanol yield (g/g)} = \left[\frac{\text{Ethanol, g/L}}{\text{Initial glucose, g/L}}\right] \quad (1)$$

$$\text{Fermentation efficiency (\%)} = \left[\frac{\text{Ethanol, g/L}}{\text{Glucose, g/L} \times 0.51}\right] \times 100 \quad (2)$$

$$\text{Ethanol productivity} = \frac{[\text{Ethanol, g/L}]}{\Delta t} \quad (3)$$

RESULTS AND DISCUSSION

EFB characterisation

The chemical compositions of lignocellulosic materials vary depending on plant varieties, geographical condition, harvesting and processing methods. The pulverized EFB was characterised (Table 1). It consists of 73.6% holocellulose, (50.3% α-cellulose and 23.3% hemicelluloses), 23.5% lignin and 3% ash. The α-cellulose being a major composition of EFB, is a glucose-based polymer which has potential for the conversion into biofuel and value-added products e.g. bioethanol. The lignin content in EFB i.e. 23.5% corresponds well with other findings [11, 12, 13, 14]. However, it is higher compared with other biomass e.g. 17-19% in rice straw [15], 6.3-9.8% in barley straw [16], 16.1% in winter rye and 14.2% in oilseed rape [17]. Thus, the need for applying a delignification step in this study.

Optimisation of acid pretreatment

In this study, EFB was hydrolysed with sulphuric acid (H_2SO_4) at varying concentrations, reaction times and temperatures. The H_2SO_4 was used considering its effectiveness for degradation of hemicellulose as compared to hydrochloric acid, nitric acid and phosphoric acid [18]. The results showed that the maximum xylose production was 277.3 mg g^{-1} when EFB was treated with 1.0% (v/v) H_2SO_4 at 125°C, for 90 min, which corresponded to 95.3% of xylose removal (Table 2). In general, the higher the pretreatment temperature and the shorter the residence time, a higher soluble xylose can be recovered [19]. At a lower temperature i.e. 105°C employing lower H_2SO_4 concentration i.e. 0.5-1.0% (v/v), only xylose was released. The xylose yields in this study were relatively higher compared to those reported i.e. 135.94 mg g^{-1} employing optimum conditions of 0.8% (v/v) H_2SO_4 at 190–210°C [20]. This implied that H_2SO_4 in very low concentration is able to hydrolyse hemicellulose of EFB to simple sugar i.e. xylose at a relatively lower temperature. Compositional analysis showed 23.6% of total hemicellulose, while 10.7% of lignin and 2.4% of ash had been removed in this pretreatment step, thus increasing the proportion of cellulose to 81.2% (Table 1).

Table 1. Empty fruit bunches properties before and after pretreatment.

Chemical compositions	Percentage (wt %, dry basis)	
	Raw EFB	Pretreated EFB
Holocellulose	73.57 ± 1.41	86.67 ± 2.01
α-cellulose	44.53 ± 0.06	81.23 ± 5.03
Hemicellulose	29.05 ± 1.48	5.45 ± 3.02
Lignin	23.45 ± 1.48	12.76 ± 2.18
Ash	2.98 ± 0.07	0.57 ± 0.17

Table 2. Effect of different variables (acid concentration, time, temperature) on the release of sugars during acid pretreatment of empty fruit bunches.

Time (90 min)				
Temperature (°C)	**Acid concentration (%, v/v)**	**Xylose yield (mg g^{-1} dry substrate)**	**Glucose yield (mg g^{-1} dry substrate)**	**Xylose removal (%)**
105	0.5	93.5 ± 6.35	n.d	32.13
	1.0	180.3 ± 4.69	n.d	61.96
	2.0	226.5 ± 5.69	10.6 ± 0.45	77.83
	3.0	220.2 ± 25.8	12.5 ± 1.04	75.67
125	0.5	269.2 ± 7.98	12.9 ± 0.68	92.51
	1.0	277.3 ± 7.01	20.5 ± 0.46	95.29
	2.0	254.6 ± 9.25	28.2 ± 2.12	87.49
	3.0	234.7 ± 2.89	34.2 ± 0.95	80.65

Time (120 min)				
Temperature (°C)	Acid concentration (%, v/v)	Xylose yield (mg g^{-1} dry substrate)	Glucose yield (mg g^{-1} dry substrate)	Xylose removal (%)
105	0.5	139.6 ± 14.54	n.d	47.80
	1.0	215.9 ± 10.77	n.d	74.19
	2.0	245.8 ± 6.50	12.4 ± 0.21	84.46
	3.0	243.0 ± 5.96	14.5 ± 5.57	83.51
125	0.5	262.3 ± 1.96	15.9 ± 0.10	90.14
	1.0	261.6 ± 2.82	23.0 ± 0.75	89.90
	2.0	244.3 ± 0.32	33.7 ± 0.76	83.95
	3.0	216.0 ± 5.59	44.3 ± 1.58	74.27

*n.d- not detected; hemicellulose content: 29.1%

EFB delignification

The presence of lignin impedes enzymatic hydrolysis of carbohydrates by blocking access of cellulose and irreversibly binding hydrolytic enzymes. Negative correlation between the percentage of lignin in plant material and its enzymatic digestibility [21], thus the lignin removal is essential in order to improve the enzymatic hydrolysis of EFB. Three delignifying agents were selected i.e. sodium hydroxide (NaOH), sodium chlorite ($NaClO_2$) and hydrogen peroxide (H_2O_2) at 2.0% (v/v) concentration. NaOH was found to be the most effective agent as it showed the highest percentage of delignification (41%) as compared to other chemicals tested i.e. $NaClO_2$ (20%) and H_2O_2 (11%) (Fig.1). Thus, NaOH-treated EFB was selected for subsequent enzymatic hydrolysis.

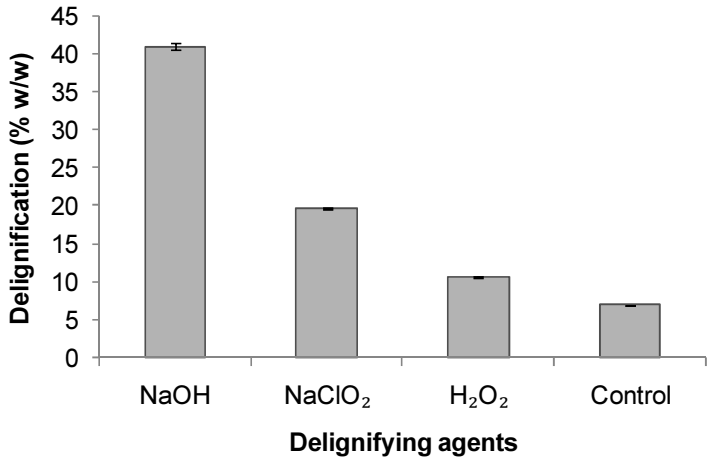

Fig. 1. Delignification of acid pretreated empty fruit bunches with different delignifying agents.

Enzymatic hydrolysis

Hydrolysis of cellulose to glucose catalysed by the cellulase enzyme suffers from slow reaction rates due to highly crystalline structure of cellulose which makes the penetration of enzymes to the active sites very difficult [18]. Addition of surfactants during enzymatic hydrolysis is capable of modifying the cellulose surface property and minimizing the irreversible binding of cellulase on cellulose [22]. Non-ionic surfactants is reported to be more suitable for enhancing the cellulose hydrolysis. Therefore, Tween 20, Tween 80 and Triton X-100 were selected and applied in this study. Table 3 shows the glucose yield during 72 h of enzymatic hydrolysis. The longer the saccharification time, the better in glucose yield throughout the saccharification period. The highest saccharification yield of 484.79 ± 0.65 mg g^{-1} of glucose was achieved in the NaOH-pretreated EFB for 72 h, which was about double the content in the control (Table 3). However, addition of Triton X-100 gave the most satisfied outcome to the hydrolysis with 533.3 ± 29.9 mg g^{-1} of glucose within 72 h. The celluloase conversion with 0.5% (w/v) Triton X-100 reached 65.7%, compared to 34.4% conversion with 81.23% cellulose in 5% (w/v) of surfactant-free treated EFB. The rate of saccharification was improved by 2.4-fold using Triton X-100 as a surfactant in hydrolysis of EFB. However, in enzymatic hydrolysis, the saccharification rate decreased proportionally with time probably due to the increased resistance of the substrate to hydrolysis [23].

Table 3. Enzymatic saccharification of pretreated empty fruit bunches

	Saccharification time (h)	Glucose yield (mg g^{-1}) dry substrate	Saccharification rate (mg g^{-1} h^{-1})	Cellulose conversion (%)
NaOH-treated	24	332.88 ± 1.59	13.87	40.98
	48	442.59 ± 22.75	9.22	54.49
	72	484.79 ± 0.65	6.73	59.68
Control	24	167.13 ± 15.38	6.96	20.57
	48	233.34 ± 21.69	4.86	28.73
	72	279.34 ± 27.34	3.88	34.39
Tween 20	24	344.27 ± 20.01	14.34	42.38
	48	446.27 ± 7.42	9.29	54.94
	72	487.73 ± 23.45	6.77	60.04
Tween 80	24	298.67 ± 22.24	12.44	36.77
	48	382.27 ± 22.89	7.96	47.06
	72	415.33 ± 23.19	5.76	51.13
Triton X-100	24	396.33 ± 11.94	16.51	48.79
	48	506.73 ± 21.52	10.55	62.38
	72	533.33 ± 29.90	7.41	65.66

Process condition: 5.0% (w/v) of empty fruit bunches and 30 FPU of cellulase, cellulose content: 81.23%

Morphological study of EFB structure

Some of the morphological changes taking place in the lignocellulosic biomass are removal of inhibitory materials, production of cracks in the lignocellulosic fibres, and exposure of cellulosic materials by creating pores during pretreatment [24]. The combined chemical pretreatments carried out in the present work was able to remove lignin, thus creating pores that enhanced surface contact with the chemicals. This prompted an easier release of sugars from the cellulose of EFB. Initially, without chemical treatment, the EFB had a relatively rigid structure and rough surface (Fig. 2a). However, physical changes occurred during dilute acid pretreatment with the appearance of white particles (about 10 µm in size) on EFB's surfaces (Fig. 2b). The EDX analysis of these particles evidenced the presence of silica (SiO_2) (Fig. 3). After alkaline pretreatment, the exposed silica and other impurities were easily wiped off leaving some empty cavities on the fibre surfaces (Fig. 2c). The pretreatment had successfully disrupted the silicified waxy surface, hence the silicon component was disposed and removed successively. The finding corresponded well with a previous study that reported NaOH-pretreatment process could remove silicon from 7.07% to 0.86% [25]. As silica deposition in biomass cell walls acts as another physical barrier to enzymatic attack [26], an effective removal of the silica during pretreatment can enhance the digestibility of the EFB.

A combined acid and alkaline pretreatment is able to enhance the exposure of cellulose component in the EFB fiber, thus leading to an improved accessibility of the cellulose for enzymatic hydrolysis. NaOH acts as an intra crystalline swelling agent i.e. it penetrates and swells both the accessible amorphous and crystalline region of cellulose [27], hence an assisted efficient EFB pretreatment process takes place. In this study, the high temperature and pressure applied during the acid-pretreatment process exploded the fibre components, making them accessible to high temperature NaOH-pretreatment. This had resulted in an effective removal of hemicelluloses and lignin, respectively from the two processes leading to a smoother surface of the treated fibre as compared to the untreated EFB (Fig. 2b and 2c). The pretreatment had successfully broken down the linkages

between the outer surface and the internal structure of the EFB. The internal of the pretreated EFB showed a clear macrofibril structure compared to the untreated EFB. These microfibrils were separated from the initial connected structure making the cellulose fully exposed and ready for enzymatic reaction [25, 28]. Later, the cellulase attacked and broke down the cellulose, causing distortion of fibre and degradation of cellulosic materials (Fig. 2d), and eventually the cellulose was converted into fermentable sugars, primarily glucose.

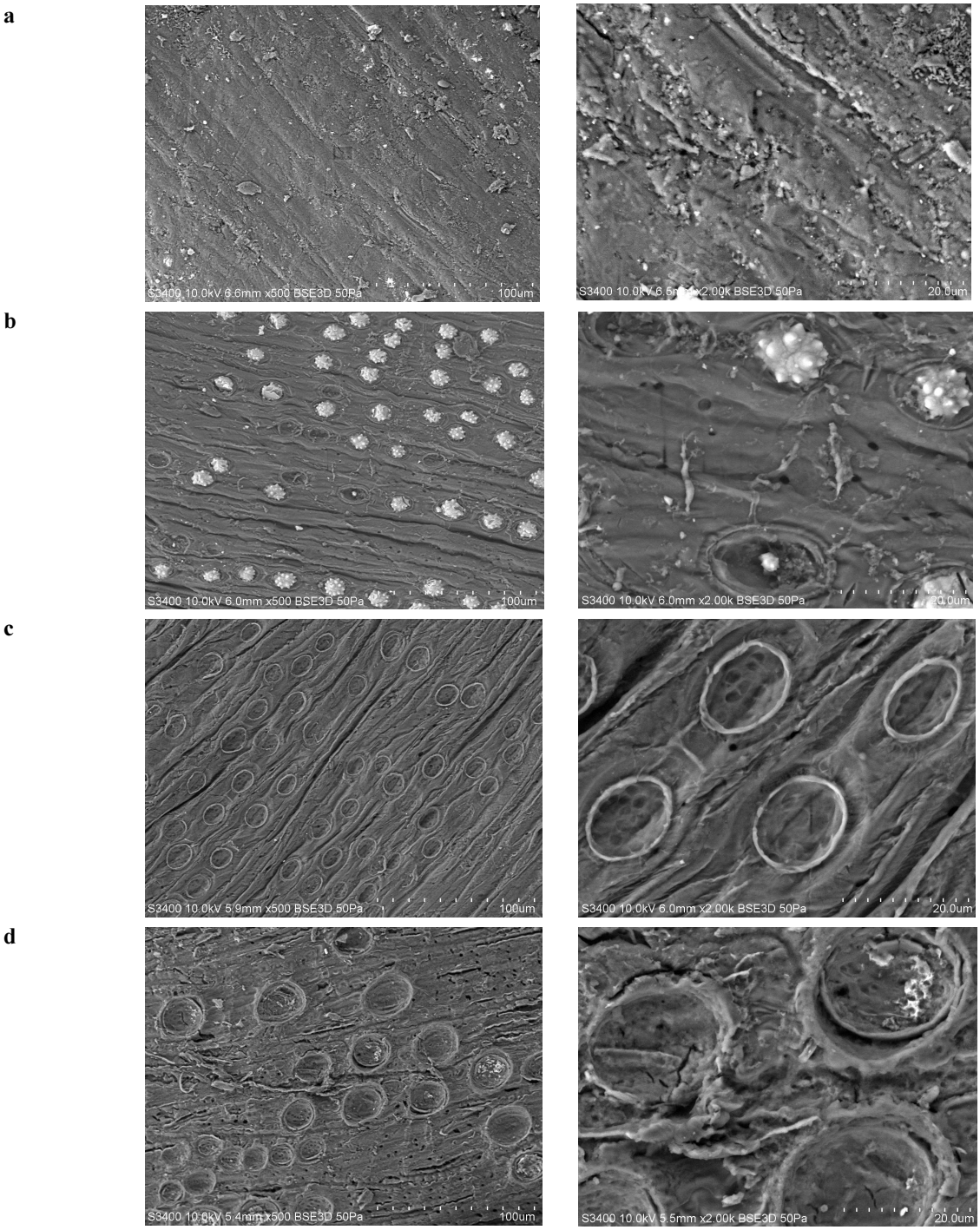

Fig. 2. Morphological changes of (a) untreated, (b) acid-pretreated, (c) alkaline-treated, (d) enzyme-treated empty fruit bunches as examined under scanning electron microscopy (SEM) at 500x and 2000x magnification.

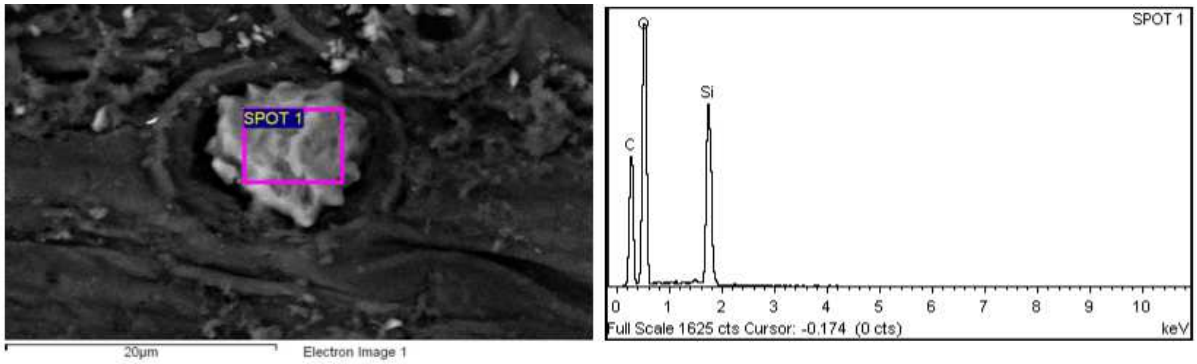

Fig. 3. The chemical compositions of the white particles of the acid-pretreated empty fruit bunches by Energy-dispersive X-ray (EDX) analysis.

Fermentation

The pretreated EFB hydrolysate was fermented using *S. cerevisiae.* Glucose consumption, bioethanol production and cell concentration were monitored at 3, 6, 24, 48 and 72 h of *S. cerevisiae* cultivation. The yeast cell adapted slowly to the medium (containing 26.7 g L^{-1} initial sugar concentration) in the beginning of fermentation and its growth increased exponentially between 3 and 24 h (Fig. 4). The bioethanol was produced started at early stage of fermentation and continuously increased and reached maximum level of 12.13 g L^{-1} within 24 h. This corresponded to bioethanol yield of 0.45 g ethanol/g glucose (Table 4). This value is comparable to that of bioethanol produced from other renewable substrates by *S. cerevisiae* i.e. oil palm trunk sap with 0.48 g/g [29]; oil palm trunk frond with 0.49 g/g [30]; sago pith residue with 0.48 g/g [31]; sugar beet molasses with 0.41 g/g [32] and sweet sorghum stalk juice with 0.39 g/g [33]. After 24 h, the cell growth was stagnant and entered the stationary phase and bioethanol production also declined slightly thereafter. This might be due to the depletion of carbon source and hence the reverted consumption of the accumulated ethanol by the organism. In this study, 89.1% of theoretical bioethanol yield was achieved with productivity of 0.51 g L^{-1} h^{-1} ferment the lignocellulosic-derived sugars from EFB to bioethanol.

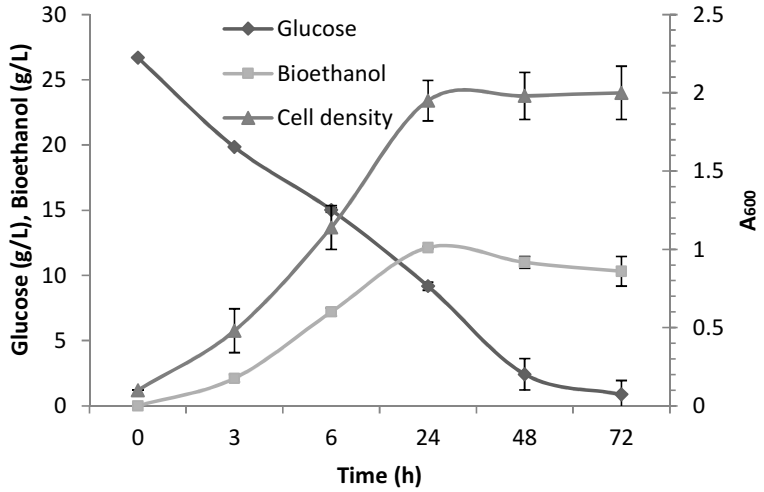

Fig. 4. Fermentation profile of the enzymatic hydrolysate of delignified empty fruit bunches by *S. cerevisiae.*

Table 4. Kinetic parameters of bioethanol fermentation by *S. cerevisiae* in empty fruit bunches hydrolysate

Time (h)	Ethanol yield, $Y_{p/s}$ (g g^{-1})	Fermentation efficiency (%)	Ethanol productivity (g L^{-1} h^{-1})
0	0.00	0.00	0.00
3	0.08	15.49	0.70
6	0.27	52.86	1.20
24	0.45	89.10	0.51
48	0.41	80.76	0.23
72	0.39	75.70	0.14

CONCLUSION

A combined chemical pretreatment of EFB - dilute sulphuric acid followed by sodium hydroxide - has effectively removed hemicellulose and lignin yielding a high cellulose. The fermentation of EFB-derived glucose obtained from the combined pretreatment is proven as good as other renewable sugars for bioethanol production. Its exploitation in biorefinery - for biobased fuels and products - can be pursued for a sustainable bioeconomy development.

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