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GLUCOSE PRODUCTION FROM STEAM-ALKALI-CHEMICAL PRE-TREATED OIL PALM TRUNK BIOMASS VIA ENZYMATIC SACCHARIFICATION PROCESS

(Penghasilan Glukosa dari Pra-Rawatan Wap-Kimia-Alkali Biomas Batang Kelapa Sawit Melalui Proses Sakarifikasi Enzim)

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Abstract

This study proposed lignocellulosic oil palm trunk (OPT) biomass can be used as alternative substrate for glucose emanation *via* enzymatic saccharification route. The OPT biomass was pre-treated using steam-alkali-chemical (SAC) method prior to enzymatic saccharification process for glucose formation. Three basic physiochemical parameters such as enzyme concentration (20 – 100 FPU.mL^{-1}), pH (4.0 – 8.0) and reaction temperature (30 – 60 °C) were intensively studied. Results revealed that all parameters gave significant effect on glucose production. By setting the enzyme hydrolysis conditions at pre-determined parameters points, i.e. enzyme concentration, 60 FPU.mL^{-1}; pH, 6.0 and reaction temperature, 50 °C, respectively; 4.27 g.L^{-1} of glucose was attained at 72 hours hydrolysis. There is 4.49-fold of glucose increment in SAC-treated OPT substrate compared to untreated ones. This research also indicates enzyme digestibility can be enhanced by using treated OPT as substrate.

Keywords: oil palm trunk biomass, steam-alkali-chemical pre-treatment, enzymatic saccharification, cellulase enzyme

Abstrak

Kajian ini mencadangkan lignoselulosa biomas batang kelapa sawit (OPT) boleh digunakan sebagai alternatif substrat bagi penghasilan glukosa melalui kaedah enzim sakarifikasi. Biomas OPT terlebih dahulu dirawat dengan wap-kimia-alkali (SAC) sebelum proses sakarifikasi enzimatik bagi pembentukan glukosa. Tiga fizikokimia parameter asas seperti kepekatan enzim (20 – 100 FPU.mL^{-1}), pH (4.0 – 8.0) dan suhu tindak-balas (30 – 60 °C) telah dikaji secara intensif. Hasil kajian menunjukkan bahawa semua parameter memberi kesan ke atas pengeluaran glukosa. Dengan menetapkan syarat enzim hidrolisis pada parameter yang telah ditentukan, iaitu masing-masing; kepekatan enzim, 60 FPU.mL^{-1}; pH, 6.0 dan suhu tindak-balas, 50 °C; 4.27 g.L^{-1} glukosa telah dicapai pada 72 jam hidrolisis. Glukosa meningkat sebanyak 4.49 kali ganda dalam SAC-rawatan OPT substrat berbanding yang tidak dirawat. Kajian ini juga membuktikan penghadaman enzim boleh dipertingkatkan dengan menggunakan OPT terawat sebagai substrat.

Kata kunci: biomas batang kelapa sawit, pra-rawatan wap-kimia-alkali, sakarifikasi enzimatik, enzim selulase

Introduction

Glucose is one of the most important monosaccharide with the chemical formula of $C_6H_{12}O_6$ and a molar mass of 180.16 g.mol^{-1}. It is the simplest form of sugar and serves as a main carbon source to living organisms including animals, human, plants and microbes for energy and metabolic requirement. Recently, glucose is produced from

lignocellulosic biomass waste such as sugarcane bagasse [1], corn stover [2] and oil palm [3]. This is because lignocellulosic biomass waste contains a higher amount of cellulose and hemicellulose which are able to be converted into sugars (mainly glucose). The produced glucose can be used as a cheaper alternative to feedstock for industrial applications particularly in fermentation industry. Usually, cellulase enzyme is required and employed to achieve the convertible goal. The efficiency of enzyme conversion towards its substrate depends on the enzymatic saccharification system. This means that the system has to provide optimal conditions for enzyme to digest its substrate in order for maximum product formation to occur. In line with this, fundamental physiochemical parameters such as enzyme concentration, pH and reaction temperature must be well manipulated [4].

However, the native behavior of recalcitrant lignocellulosic biomass makes the enzyme hydrolysis more challenging. In order to maximize sugar formation from lignocellulosic substrate, pretreatment on lignocellulosic biomass is inevitable. Apparent objectives of the pretreatment are i) to alter the structure of lignocellulose so as to make cellulose more accessible to cellulase and ii) to shatter the lignin seal and disrupt the crystalline structure of cellulose [5]. Therefore, the pretreatment step is crucial as it can maximize cellulose recovery from lignocellulosic substrate into usable form. Previous study has shown that microwave-alkali (Mw-A) pretreatment is capable of reducing lignin in small quantity. In contrast, steam-alkali-chemical (SAC) which is a double-step pretreatment method has proven to be more efficient in terms of lignin removal. According to Lai et al. [6], a significant decrease in the amount of lignin i.e. 87.07% was seen when using SAC method; whereas reduction of lignin using Mw-A pretreatment method was recorded to be only 15.33%. Lignin removal is essential since the presence of lignin will act as a shield that prevents cellulose from being hydrolyzed by cellulolytic enzymes. Thus, the double-step pretreatment (i.e. SAC method) was selected and used to pre-treat the OPT substrate in this study.

Currently, Malaysia is recognized as the world's second largest palm oil producers after Indonesia [7]. The oil palm biomass waste has amounted to 59 million tons annually which include (dry weight): oil palm empty fruit brunches (OPEFB), oil palm frond (OPF) and oil palm trunk (OPT), respectively. To eliminate bulk oil palm biomass in a more economic way; one of the method is to convert the oil palm biomass into fermentable sugars. The formed sugars (mainly glucose) can be utilized as carbon feedstock by microbes to produce the desired fermentative products such as organic acids, bio-ethanol and so on. The present study has chosen oil palm trunk (OPT) biomass as the substrate candidate. This is due to limited research data that focused on oil palm trunk studies compared to other parts of oil palm such as oil palm frond, oil palm empty fruit bunch and palm kernel shell. [8]. In order to maximize lignin removal and cellulose recovery, steam-alkali-chemical (SAC) pre-treatment method is used to accomplish the objectives. There are three important parameters to be considered for glucose conversion under enzymatic saccharification process, namely: enzyme concentration, pH and reaction temperature and all was investigated thoroughly. Apart from that, the findings of this research can provide complementary research data to oil palm study.

Materials and Methods

Raw materials

The pulverized oil palm trunk (OPT) biomass was provided by Concept Renewable Energy Sdn. Bhd., Malaysia. It was sieved to a particle size of less than 1.0 mm and dried at 45 °C for 3 days [7].The OPT samples were kept in 4 °C chiller prior to pre-treatment and enzymatic hydrolysis.

Chemical reagents and enzymes

The chemicals used in the experiment were of analytical grade with highest purity, i.e. 96 – 99% and purchased from Merck (M) Sdn. Bhd. namely: sodium hydroxide (NaOH), acetic acid (CH_3COOH), potassium sodium tartrate ($KNaC_4H_4O_6{\cdot}4H_2O$), sodium metabisulfite ($Na_2S_2O_5$), phenol (C_6H_6O), sodium azide (NaN_3) and 3,5-dinitrosalicylic acids. The household bleach (sodium hypochlorite solution, 5.75% active chlorine) was bought from Tesco hypermarket. Meanwhile, the cellulase enzyme produced from *Trichodermareesei*, citric acid monohydrate ($C_6H_{10}O_8$) and trisodium citrate dehydrate ($C_6H_9Na_3O_9$) were obtained from Sigma-Aldrich (M) Sdn. Bhd.

Steam-alkali-chemical (SAC) pre-treatment method

Ten grams of oil palm trunk (OPT) sample was soaked in a beaker containing 200 mL of 2.5 M NaOH solution for 1 hour. The mixture was brought to autoclave at temperature, 121 °C; reaction time, 20 min and pressure, 1.224 kg-

force.cm^{-2}, respectively [6]. Upon completion, the aqueous NaOH solution was discarded and the OPT slurry was washed with tap water (4 x 1000 mL) followed by distilled water (4 x 1000 mL). The steam alkaline slurry was then added with 200 mL of NaClO. The pH of the mixture was adjusted to acidic condition at pH 3.5 using CH_3COOH [9]. The acid solution was then filtered-off and washed again with tap water (4 x 1000 mL) followed by distilled water (4 x 1000 mL). The SAC treated oil palm trunk sample was freeze-dried and kept at 4 °C for enzymatic saccharification reaction. The content of the chemical constituents such as cellulose, hemicellulose, lignin and extractives of raw and treated OPT substrate is presented in Table 1.

Table 1. Chemical composition of raw and treated oil palm trunk biomass (g per 100 g biomass)

Pretreatment	(g per 100 g biomass)			
Type	Cellulose	Hemicellulose	Lignin	Extractives
Raw[7]	50.78 ± 1.05	30.36 ± 1.10	17.87 ± 1.68	0.99 ± 1.73
Mw-A[7]	71.88 ± 2.55	11.77 ± 2.30	15.13 ± 0.40	1.22 ± 0.16
SAC[9]	42.70 ± 0.57	52.80 ± 1.36	1.90 ± 0.04	2.60 ± 0.76

All measurements were duplicated and fall within the relative percentage difference below 5% [10].
Mw-A: Microwave-alkali, SAC: Steam-alkali-chemical

Cellulase enzyme activity

The cellulase enzyme activity was measured according to Ghose's protocol [11]. One unit of filter paper unit (FPU) was defined as the amount of enzyme which produced 2.0 mg of glucose from 50 mg of filter paper at temperature, 50 °C; pH, 4.8 and reaction time, 60 min, respectively. The cellulase activity obtained from this assay was 104.42 FPU.ml^{-1}.

Enzymatic hydrolysis and glucose analysis

The enzymatic saccharification reaction was performed in a glass jar and the reaction temperature was manipulated using water bath. 1 % (w/v) of SAC-treated OPT was discharged into a glass jar containing 20 mL of 50 mM sodium citrate buffer and 100 µL of 2 % of NaN_3. In the enzymatic hydrolysis study, physiochemical parameters such as enzyme concentration (20 – 100 FPU.mL^{-1}), pH (4.0 – 8.0) and reaction temperature (30 – 60 °C) were tested using one-a-time variable method. For instance, when enzyme concentration analysis was carried out, both pH and temperature were set to 4.8 and 50 °C, respectively. The pre-set constant values were set according to the manufacturer's recommendation [12]. Once the optimal conditions such as enzyme concentration, pH and temperature were pre-determined; these selected conditions were used to re-run the enzymatic saccharification on untreated and SAC-treated OPT.

For all enzymatic study, exactly 200 µL of enzyme hydrolysate was withdrawn and deposited into microtube at specific intervals. The sample was mixed with DNS reagent (ratio of sample and DNS = 1:1), heated and agitated at 100 °C and 400 rpm, respectively for 15 min on a thermoblock. The brown color solution was used to determine glucose concentration at 540 nm using spectrophotometer (Thermo, Genesys 20). The reducing sugars derived from enzyme hydrolysis were considered as glucose equivalent [13]. All hydrolysis experiment was performed in triplicate and error was within ± 5% [14].

Results and Discussion

Effect of different enzyme concentration on glucose production

In this study, cellulase concentrations (20 – 100 FPU.mL^{-1}) were tested at fixed pH, 4.8; reaction temperature, 50 °C and substrate mass, 1% (w/v), respectively. Figure 1 illustrates the 72 hours' time profile of glucose formation derived from saccharification process.

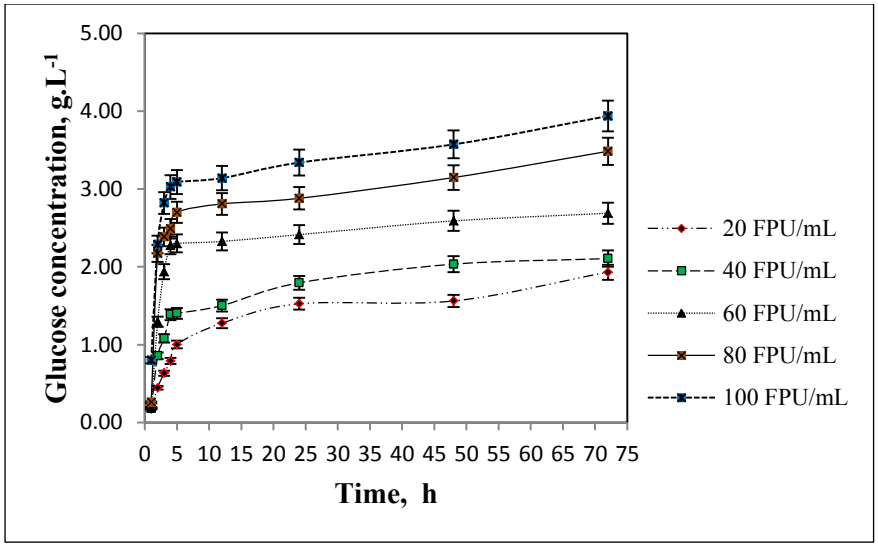

Figure 1. Effect of different enzyme concentration (20 – 100 FPU/mL) on glucose production

After the 72 hours enzyme saccharification, a glucose concentration of 1.93 $g.L^{-1}$ was recorded at 20 $FPU.mL^{-1}$ enzyme concentrations. When the enzyme amount increases from 40 – 100 FPU/mL, glucose concentration also increased to 2.11 – 3.94 $g.L^{-1}$ with an increment value of 9.32 – 104.15%. This indicates that formation of glucose amount in the present study could be enhanced by supplying a higher dose of cellulase enzyme. When more cellulase is supplied, there will be extra active sites available for substrate (present study: substrate is SAC-treated OPT). Hence, it will be easier for substrate to bind to the enzymes' active sites to form enzyme-substrate complex. This will eventually accelerate the enzymatic reaction and thus more glucose is released.

Although, the maximum glucose concentration was attained at 100 $FPU.mL^{-1}$; 60 $FPU.mL^{-1}$ of cellulase concentration was selected for the subsequent enzymatic study. This is because the cellulase enzyme cannot be recycled and a higher dose of enzyme usage will contribute to higher operation cost. Moreover, previous studies which demonstrated the usage of cellulase enzyme in enzymatic saccharification on lignocellulosic biomass for glucose production, utilized enzyme concentrations within the range of 20 – 60 $FPU.mL^{-1}$ [15-17]. Based on these views, enzyme concentration at 60 $FPU.mL^{-1}$ was selected and used for the next experiment.

Effect of different pH on glucose production

In this investigation, different pH values (pH 4.0 – 8.0) were tested on pre-selected enzyme concentration (60 $FPU.mL^{-1}$), reaction temperature (50 °C) and 1% (w/v) of SAC-treated OPT substrate for glucose production. Figure 2 depicts glucose concentration increased from strong to weak acidic condition (pH 4.0 – 6.0), i.e. 1.27 to 4.03 $g.L^{-1}$. There is about 3.17-fold of glucose increment when compared to pH 6.0 and pH 4.0. On the other hand, at neutral and weak alkaline pH conditions (i.e. pH 7.0 and pH 8.0), the glucose concentration slightly declined compared to pH 6.0, *viz.*10.92 – 11.66 % or equivalent to 3.56 and 3.59 $g.L^{-1}$, respectively. In general, the ionic group of enzyme reacts well within suitable pH ranges either in acidic or alkaline conditions. The variation of pH causes modification on enzymes' ionic groups and its three-dimensional structure [18]. The changing of enzyme structure or shape makes substrate unfit to enzymes' active sites and thus reduces the chances of enzyme-substrate complex formation. This can directly impede the rate of enzymatic reaction.

Current study revealed that cellulase enzyme is active in weak acidic and weak alkaline pH range (pH 6.0 – 8.0). The stronger acidic pH conditions (< pH 6.0) did not favour the cellulase hydrolysis. This is the reason for getting lower glucose content at pH 4.0 – 5.0 compared to pH 6.0 – 8.0. It is obvious that the optimal pH point is pH 6.0 and it was chosen for subsequent study.

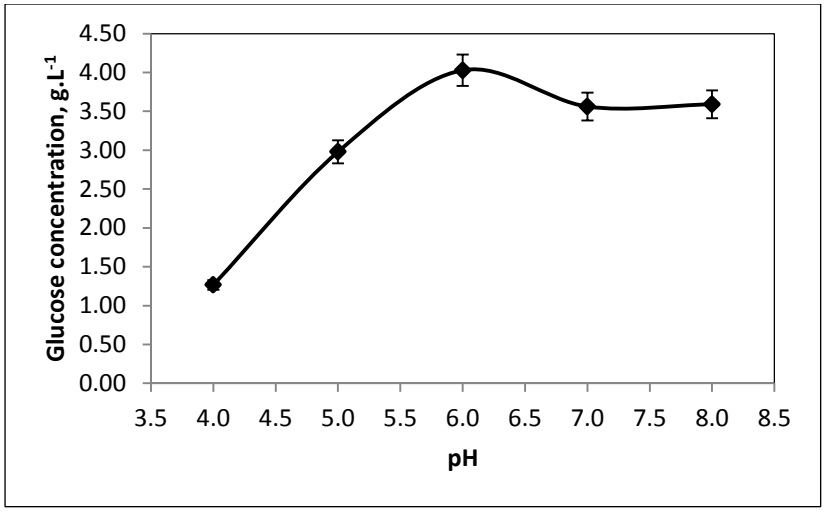

Figure 2. Effect of different pH (4.0 – 8.0) on glucose production

Effect of different temperature on glucose production

The range of temperatures, 30 to 60 °C was tested for glucose production by using 1% (w/v) of SAC-treated OPT sample. Other parameters such as enzyme concentration and pH were pre-set according to previous studies, i.e. 60 $FPU.mL^{-1}$ and pH 6.0, respectively.

Figure 3 demonstrates the glucose production from cellulase-SAC OPT hydrolysis at different temperature points. It is observed that glucose amount increased from 2.01 to 3.68 $g.L^{-1}$ when reaction temperatures rise from 30 to 50 °C. The total increment is up to 83.08%. The possible explanation is that the enzyme-catalysed reaction like most chemical reactions; proceeds at a faster velocity when temperature is increased. The temperature rise during enzymatic saccharification process imparts more kinetic energy to reactant molecules, resulting in more productive collision per unit time [19]. Consequently, more glucose is released when the system temperature was boosted from 30 to 50 °C.

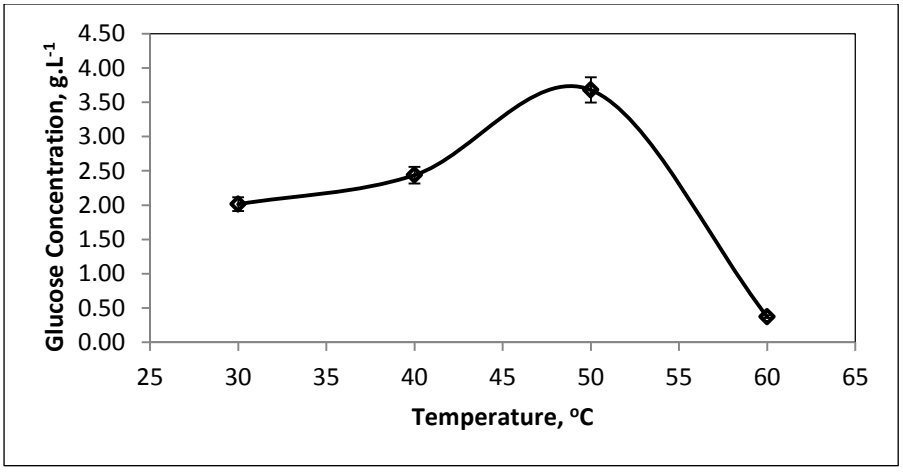

Figure 3. Effect of different temperature (30 – 60 °C) for glucose production

On the other hand, only 0.37 $g.L^{-1}$ of glucose concentration was obtained at 60 °C. This is in line with the fact that enzyme molecules possess delicate structure. The disruption of enzyme tertiary structure can occur when enzyme is exposed to temperature beyond its optimal point (present study; temperature optimal point = 50 °C). Similar phenomenon is observed in hydrolysis of cellulase-Mw-A treated OPEFB substrate where cellulase displayed deactivation when the reaction temperature is beyond 50 °C [20]. As shown in Figure 3, enzyme is considered to be partially denatured as it lose its catalytic power when temperature > 50 °C. This explains why reaction temperature of 60 °C gained less glucose amount compared to reaction temperature of 30 and 40 °C, respectively. Based on Figure 3, the optimal hydrolysis temperature point is 50 °C and it was selected for subsequent enzymatic experiment.

Comparison of untreated and SAC-treated OPT under pre-determined enzymatic conditions

The comparison of untreated and SAC-treated OPT sample was performed under pre-determined enzymatic conditions where enzyme concentration, pH and reaction temperature were set to 60 $FPU.mL^{-1}$, 6.0 and 50 °C, respectively. The amount of both substrates used in this study was 1% (w/v). After the 72 hours enzyme hydrolysis, up to 4.27 $g.L^{-1}$ glucose concentration was obtained in SAC-treated OPT; whereas the untreated OPT sample only produced 0.95 $g.L^{-1}$ of glucose (as shown in Figure 4). The glucose increased 4.49-fold when using the SAC-treated OPT biomass as a substrate.

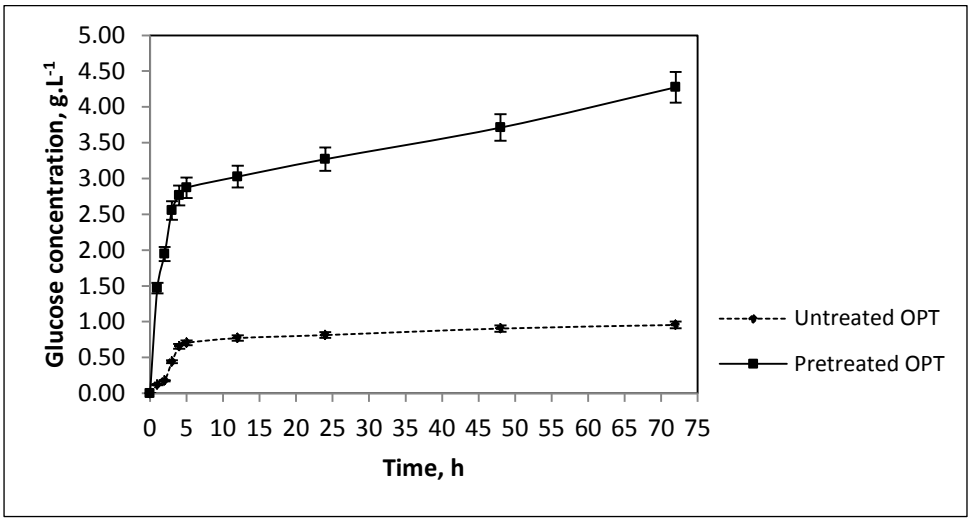

Figure 4. Glucose production for untreated and SAC-treated OPT under pre-selected enzyme hydrolysis conditions

The SAC pre-treatment process has enhanced the enzyme digestibility towards its substrate. This is attributed to disruption of encrusted lignocellulosic OPT structure by loosening the complex linkage among the cellulose, hemicellulose and lignin. Hence, more amorphous cellulose is released and readily available for cellulase enzyme. Binod et al. stated that treated lignocellulosic biomass contain more amorphous cellulose whereas untreated biomass contain more crystalline cellulose and the former is easily digested by cellulose [21]. In short, SAC pre-treatment process can effectively enhance the enzymatic saccharification rate in this study.

A recent report revealed that 72 hours enzymatic hydrolysis on SAC treated oil palm trunk biomass produced 16.59 $g.L^{-1}$ of glucose [9]. In contrast, the present study which had used a single enzyme (cellulase) to accomplish the hydrolysis process only produced 4.27 $g.L^{-1}$ of glucose at 72 hours. The main reason for the significant difference (glucose concentration) between these works is the use of an additional enzyme, i.e. addition of β-glucosidase in hydrolysis reaction. The existence of β-glucosidase not only improves enzymatic hydrolysis performance but also expedites the enzymatic saccharification rate. Therefore, more glucose is formed in the former study. However,

when using two enzymes in hydrolysis reaction, the glucose production cost will dramatically increase. Moreover, the cellulolytic enzymes cannot be recycled. Since one of the objective is to reduce the operational cost, this study only employed cellulase enzyme in the saccharification reaction for glucose production.

In another study, Hamzah et al. had conducted 24 hours' enzyme hydrolysis on Mw-A treated OPEFB [20]. The glucose amount of 0.49 $g.L^{-1}$ and 0.72 $g.L^{-1}$ were recorded for single enzyme (cellulase) and two enzymes (cellulase + β-glucosidase), respectively. Comparatively, 3.27 $g.L^{-1}$ amount of glucose was formed at 24 hours (see Fig. 4); which is 4.51 to 6.67-fold higher than previous study. This explains that SAC-treated substrate with its lower lignin content has more advantage compared to Mw-A treated substrate. The double-step pre-treatment outperformed single-step pretreatment in lignin removal. Lignin behaves like a protected shield and avoids cellulose from being hydrolyzed by cellulase. Through harsher pre-treatment method (like SAC conditions), lignin is removed effortlessly and more amorphous cellulose is exposed. Consequently, enzyme digestibility towards SAC OPT substrate is enhanced and resulted in more glucose formation.

Conclusion

Three physiochemical parameters namely: enzyme concentration, pH and reaction temperature were tested in this investigation. Results divulged that optimal conditions for enzyme hydrolysis on steam-alkali-chemical (SAC) treated OPT were 100 $FPU.mL^{-1}$ of enzyme concentration, pH 6.0 and reaction temperature at 50 °C, respectively. However, 60 $FPU.mL^{-1}$ of cellulase was chosen in the subsequent enzymatic study as to reduce the operational cost for glucose production. A 4.49-fold of glucose increment was attained in enzymatic saccharification on SAC-treated OPT compared to untreated ones. In conclusion, SAC pretreatment can be potentially scaled up for industrial applications. The study also suggested that oil palm trunk biomass can be a cheaper alternative of substrate for high value added products such as glucose.

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