Effect of Ultrasound-Assisted on Sequential Peracetic Acid and Alkaline Peroxide Pretreatment to The Enzymatic Hydrolysis of Oil Palm Empty Fruit Bunch

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Research

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Abstract

Enzymatic hydrolysis of oil palm empty fruit bunch (OPEFB) that has been pretreated by modified pretreatment has been investigated in this study. The OPEFB used was pretreated by using sequential peracetic acid – alkaline peroxide solution. As the modification method, the assistance of pretreatment by ultrasound was conducted, in order to increase the enzyme accessibility. Therefore, it enhances the production of reducing sugar on the hydrolysis process. Prior to hydrolysis process, OPEFB was initially treated by using peracetic acid solution, comprise of CH_3COOH (> 99%) and H_2O_2 (30% w/w), assisted by ultrasound for 3 hours at 35°C. Afterwards, OPEFB was treated by using alkaline peroxide solution, comprise of NaOH (40% w/w) and H_2O_2 (35% w/w), assisted by ultrasound for 10 hours at 35°C. OPEFB that has been pretreated was then subjected to enzymatic hydrolysis process using cellulase enzyme, in order to convert cellulose content into reducing sugar. Enzymatic hydrolysis was carried out at 50°C in a shaker incubator with 150 rpm for 48 hours. In this study, the effect of different enzyme concentration and hydrolysis time towards the sugar concentration in modified-pretreated OPEFB was observed and analyzed. Three different concentrations of enzyme were used, including 1.25, 2.5, and 5 g/L, and reducing sugar concentrations were analyzed at 30 and 45 minutes, and 1, 2, 4, 6, 24, 30, and 48 hours. Based on results, enzyme concentration has a significant effect to the production of reducing sugar. The reducing sugar concentrations obtained at the end of the hydrolysis process were 8.48, 11.06, 19.16 g/L, at the enzyme concentrations of 1.25, 2.5, and 5 g/L, respectively. At any hydrolysis time, the highest sugar concentration has been achieved on the highest enzyme concentration of 5 g/L. Moreover, the effective hydrolysis time were achieved at 6 hours, at all concentration of enzyme, since the production of reducing sugar were insignificant after 6 hours. This study showed an increase in reducing sugar production by 8.25% in the hydrolysis process using OPEFB pretreated by modified pretreatment compared to the non-modified pretreatment.

Introduction

Recent trends on the utilization of lignocellulose biomass has been risen due to its cellulose content which is potential to be converted into various valuable chemical products, such as biofuel and fine chemical (Walker 2010; Wyman 1999). Lignocellulose biomass are refer to all natural plants and materials that contain high carbohydrate content such as agricultural and forest residues, industrial and municipal wastes (Walker 2010). The carbohydrate content on lignocellulose biomass are composed by cellulose fiber interlock by hemicellulose and lignin (Wyman 1999). The cellulose and hemicellulose content can be hydrolyzed into reducing sugar that can be converted into various valuable chemical products through chemical process or fermentation. Among various types, one of promising lignocellulose biomass is oil palm empty fruit bunch (OPEFB), since it has high cellulose content that can be converted into fermentable sugar. OPEFB typically comprises of 17–33% hemicellulose, 43–65% cellulose and 13–37% lignin on a dry weight basis (Khalil et al. 2012). Furthermore, OPEFB also abundantly generated throughout the world. Around 37.7 million tons of OPEFB is generated annually

with limited uses (Cui et al. 2014; Sumathi et al. 2008). Therefore, OPEFB is potential and promising feedstock to produce high value chemical products.

Unfortunately, the conversion of cellulose and hemicellulose on OPEFB into fermentable sugar usually faces several obstacles. One of the obstacles is the existence of lignin content on the biomass which can block the cellulose and hemicellulose conversion into fermentable sugar on the hydrolysis process. Therefore, prior treatment before hydrolysis for delignification should be conducted to remove lignin content on the biomass. Without any pretreatment, hydrolysis of cellulose and hemicellulose into simple sugars will be unsuccessful, as lignin has an ability to prevent the hydrolysis agent, such as enzyme, accessing the cellulose and hemicellulose (Hendriks and Zeeman 2009).

Several pretreatments have been developed for delignification of lignocellulose biomass (Mussatto and Roberto 2004; Sklavounos et al. 2013; Tan et al. 2013), either using acids, alkalis or organic solvents as the chemical agents (Zhao et al. 2009). Generally, acid pretreatment has an objective to change the structure of lignin obtaining the acid soluble lignin (Mosier et al. 2005) and solubilize the hemicellulose fraction (Alvira et al. 2010). Meanwhile, alkali pretreatment will destroy the ester linkages between lignin and carbohydrates (Sun and Cheng 2002), remove nearly all lignin and a part of hemicellulose that will improve the enzymatic hydrolysis of cellulose into sugars (Taherzadeh and Karimi 2008). On the other hands, organic solvents helps to solubilize the network of lignin and portion of hemicellulose (Pan et al. 2006; Taherzadeh and Karimi 2008), destroy the lignin, lignin-hemicellulose, and glycosidic bonds (Zhao et al. 2009).

Moreover, on several treatments, the chemicals are combined with oxidizing agents such as hydrogen peroxide and sodium hypochlorite (Nazir et al. 2013) to helps delignification and depolymerization processes. Several studies have investigated the delignification of OPEFB using acid and alkali combined with hydrogen peroxide. OPEFB has been successfully delignified by using NaOH and H₂O₂, both simultaneously and consecutively, with lignin removal of 72% and 99% (Misson et al. 2009). On the other hand, pretreatment of OPEFB using peracetic acid solution, composed by glacial acetic acid and hydrogen peroxide, has also been investigated obtaining 53% of lignin removal with nearly all hemicellulose was retained (Palamae et al. 2014). Furthermore, sequential treatment using peracetic acid solution and alkaline peroxide solution on OPEFB obtaining lignin removal around 92% (Palamae et al. 2017).

Other than using chemical treatment, improvement of biomass pretreatment can be achieved by using physical treatment. Various physical treatment has been studied to improve delignification of biomass, such as using microwave or ultrasound treatment. Pretreatment of OPEFB using microwave had been studied by using a sequential dilute acid and microwave alkaline pretreatment, obtaining high delignification and high amount of cellulose (Akhtar and Idris 2017). On the other hand, a pretreatment assisted by ultrasound has been investigated using dilute aqueous ammonia on corn cob, corn stover and sorghum stalk as the biomass. Based on this study, the assistance of ultrasonic pretreatment can

increase the accessibility of cellulose in the biomass and therefore increase the sugar yield on enzymatic hydrolysis process (Xu et al. 2017).

In the previous work, we have revealed an increase in cellulose content and a decrease in lignin content in OPEFB by performing a pretreatment using sequential peracetic acid and alkaline peroxide solution assisted by ultrasound (Hermansyah et al. 2019). However, the evaluation and investigation of sugar production on the enzymatic hydrolysis process using OPEFB pretreated by sequential peracetic acid and alkaline peroxide assisted by ultrasound has not been carried out. Therefore, in this present work, the modified-pretreated OPEFB will be subjected to enzymatic hydrolysis process in order to obtain the reducing sugar. Therefore, this study aims to evaluate and investigate the production of reducing sugar concentration from OPEFB that pretreated by modified pretreatment using sequential peracetic acid – alkaline peroxide solution assisted by ultrasound, under different enzyme concentration and hydrolysis time. Moreover, comparison of reducing sugar production with the non-modified pretreated OPEFB will also be examined. Hence the increase of sugar concentration can be recognized and proven.

Methods

Biomass, Chemicals, and Enzyme

Oil palm empty fruit bunch (OPEFB) was procured from a local supplier (Riau, Indonesia). It was washed, sun-dried and then dried in an oven at 70°C for 24 hours. Dried OPEFB were then ground and sieved using a sieve with a mesh number of 30- and 0.6-mm aperture. Sieved OPEFB were then stored in a container at room temperature, for further use.

For the pretreatment process, the following chemicals were used: glacial acetic acid 100%, sodium hydroxide, and hydrochloric acid 37%, procured from Merck KGaA (Darmstadt, Germany); sulfuric acid and acetone, procured from Mallinckrodt (Phillipsburg, NJ, USA); and hydrogen peroxide 35%, procured from PT. Wiloso Yasa Pratama (Jakarta, Indonesia).

For enzymatic hydrolysis, the following chemicals were used: Cellulase from *Aspergillus niger*, with approximately 29000 unit/g activity, was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan); citric acid and sodium citrate dehydrate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

For sugar analysis: pure glucose was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Benedict's reagent solution was purchased from Showa Chemical Co., Ltd. (Tokyo, Japan).

Pretreatment

Pretreatment of OPEFB was carried out by using peracetic acid and alkaline peroxide solution, sequentially. Modified pretreatments were conducted by the assistance of ultrasound, according to the previous research (Hermansyah et al. 2019).

Prior to the pretreatment process, preparation of peracetic acid and alkaline peroxide solution were carried out first. The peracetic acid (PA) solution was prepared from 600 mL of glacial acetic acid and 400 mL of hydrogen peroxide (30% w/w). Afterwards, 15 mL of sulfuric acid was added to the solution, as the catalyst. The solution was mixed for 72 hours at a room temperature of 35°C using a magnetic stirrer. Furthermore, alkaline peroxide (AP) solution was prepared from 174 mL of sodium hydroxide (40% w/w) and 26 mL of hydrogen peroxide (35% w/w) (Palamae et al. 2014).

Pretreatment of OPEFB using PA solution was carried out by mixing 50 g of OPEFB with 1000 mL PA solution (20 mL of PA solution per gram of OPEFB) in 2 L of Duran glass bottle. For modified pretreatment, it was conducted in an ultrasonic processor (53 kHz, 90W), at the temperature of 35°C for 3 hours (Hermansyah et al. 2019). However, for non-modified pretreatment, it was stirred using an overhead stirrer for 9 hours. Afterwards, the slurry was filtered using a muslin cloth. The pretreated OPEFB was then washed with distilled water, neutralized with 6 M NaOH, and then washed further using distilled water. The solids obtained were then dried in an oven at 80°C for 48 h. After drying, the pretreated OPEFB were stored in a container at room temperature, for further use (Palamae et al. 2017).

Further pretreatment using AP solution was carried out by mixing 10 g of pretreated OPEFB with 200 mL AP solution (20 mL of AP solution per gram of OPEFB) in a 500 mL Duran glass bottle. Modified pretreatment was conducted inside an ultrasonic processor (53 kHz, 90W), at the temperature of 35°C for 10 hours (Hermansyah et al. 2019). Nevertheless, non-modified pretreatment was carried out in the shaker water bath for 12 hours. Subsequently, the slurry was filtered using a muslin cloth. Pretreated OPEFB was washed with distilled water, neutralized to a final pH of 5.5–6.0 with 6 M hydrochloric acid, and washed again with distilled water. The solid fraction of pretreated OPEFB was then dried in an oven at 80°C for 48 h. Dried pretreated OPEFB were stored in a container at room temperature, for further use (Palamae et al. 2017).

Analysis of OPEFB Content

The content of OPEFB before and after pretreatment were analyzed using the methods from National Renewable Energy Laboratory (NREL), version 08-03-2012. The analysis was determined based on acetone extractable material, hemicellulose content, and acid insoluble lignin contents.

Acetone extractives were obtained by the extraction of 5 gr of OPEFB (W_0) using acetone (80 mL) in a Soxhlet extractor. The extraction was carried out at 90°C for 1 hour. Afterwards, fresh acetone was added in order to wash the OPEFB for 30 minutes. OPEFB were then air dried at room temperature and further oven dried at 105°C to a constant weight. The dried OPEFB were then cooled in a desiccator and weighed (W_1). The acetone extractable contents E (%) of the OPEFB were calculated using the Eq. 1 (Li et al. 2004):

$$E(\%) = \frac{W_0 - W_1}{W_0} \times 100\% \quad (1)$$

Hemicellulose content was determined by reacting 0.3 g dry sample of acetone extraction (W_2) with 3 mL of NaOH (0.5 mol/L) in a 15 mL test tube. The mixture was then heated at 80°C for 3.5 hours. Subsequently, the mixture was cooled to room temperature and filtered under vacuum condition. The solid residue was washed with distilled water until a pH level of 7 was reached. The residue was then oven dried at 105°C to a constant weight, and the dried residue was cooled in a desiccator and weighed (W_3). The hemicellulose content H (%) of the OPEFB fibers was calculated using Equation 2:

$$H(\%) = \frac{W_2 - W_3}{W_2} \times 100\% \quad (2)$$

The acid insoluble lignin content was determined by reacting 0.2 g dry sample of acetone extraction (W_4) with sulfuric acid (200 mL, 720 g/L), which poured gently, in a Erlenmeyer flask. The flask was then reacted at 30°C for 1 hour. Afterwards, 56 mL of distilled water was added into the flask and autoclaved at 121°C for 1 hour. After cooling, the residues were filtered using Whatman no. 1 filter paper and washed with distilled water. The washed residue was then oven dried to a constant weight at 105°C, and the dried residue cooled in a desiccator and weighed (W_5). The contents of acid insoluble lignin L (%) were calculated using Equation 3:

$$L(\%) = \frac{W_5}{W_4} \times 100\% \quad (3)$$

As acetone extractives, hemicellulose, acid insoluble lignin and cellulose are the only components found in OPEFB (*Di Blasi et al. 1999; Lin et al. 2010; Menon and Rao 2012*), the cellulose content C (%) was generally calculated from the other measured values, as follows:

$$C(\%) = 100 - H(\%) - L(\%) \quad (4)$$

Enzymatic Hydrolysis

Enzymatic hydrolysis of pretreated OPEFB, both by modified and non-modified pretreatment, were carried out in Erlenmeyer flask containing 0.2 gr of pretreated OPEFB and 10 mL of 50 mM citrate buffer solution at pH 5. Hydrolysis was performed in a shaker incubator (BioShaker, Taitec) at 50°C with 150 rpm for 48 hours. For modified-pretreated OPEFB, three different concentrations of cellulase enzyme were applied: 1.25, 2.5, and 5 g/L. Moreover, during hydrolysis process, samples were taken at 30 and 45 minutes, 1, 2, 4, 6, 24, 30, and 48 hours. Samples were then boiled to deactivate the enzyme activity and then stored in the refrigerator for further sugar analysis.

Analysis of Reducing Sugar Concentration

Concentration of reducing sugar in hydrolysis product were analyzed using Benedict's reagent method. 1 mL of hydrolysis product was taken into a reaction tube and added with distilled water into the total volume of 4.5 mL. Benedict's reagent of 0.2 mL was then added into the tube. The mixture was then homogenized using a vortex and then boiled for 10 minutes (Cochran et al. 2008). The absorbance was measured using UV-VIS spectrophotometer (UV mini 1240, Shimadzu) at 600 nm. The enzymatic hydrolysis sugar yields were then calculated using Equation 5 (Agrawal et al. 2018; Santhi et al. 2014), as the ratio of reducing sugar concentration released to the carbohydrate content of the biomass, including cellulose and hemicellulose.

$$\text{Hydrolysis yield(\%)} = \frac{\text{Concentration of reducing sugar (g)} \times 0.9 \times 100}{\text{Carbohydrate content of the biomass (g)}} \quad (5)$$

Results And Discussion

Effect of pretreatment on OPEFB

Sequential pretreatment of OPEFB using peracetic acid solution followed by alkaline peroxide solution has been carried out in this study. Two types of pretreatment were carried out in this study, including modified and non-modified pretreatment. Modified pretreatment was carried out with the assistance of ultrasound during the pretreatment process. However, non-modified pretreatment was conducted without the assistance of ultrasound.

In this pretreatment, peracetic acid solution was chosen as one of the pretreatment agent due to their ability to remove lignin content selectively from the biomass (Sundara 1998). Removal of lignin by peracetic acid solution occurs by several reaction mechanism, including hydroxylation of the aromatic rings, oxidative demethylation, oxidative ring opening, displacement of side chains, cleavage of b-arylether bonds and epoxidation (Song et al. 2013). Furthermore, peracetic acid solution has an ability to maintain hemicellulose content on the biomass, therefore the loss of hemicellulose can be reduced (Palamae et al. 2014). Moreover, the selection of alkaline peroxide as the pretreatment agent was done as it removes almost all lignin content on the biomass (Misson et al. 2009). Alkaline peroxide pretreatment can also improve the performance of enzymatic hydrolysis, since it plays a role in lignin release from the lignocellulose matrix and increases the degree of hydration of the cellulose (Gould 1985). In the other side, the assistance of ultrasound on the pretreatment will increase the accessibility of cellulose in the biomass and sugar production on enzymatic hydrolysis process will be increased (Xu et al. 2017).

Based on results, the effect of pretreatment on the physical appearance of OPEFB are depicted in the Fig. 1.

Based on physical appearance, there are significance differences between untreated and pretreated OPEFB, particularly on the color and structure of OPEFB fiber. Untreated OPEFB looks dark brown in color

with large, coarse, and strong fibers. However, pretreated OPEFB by non-modified pretreatment seen light brown, with a rather large, quite coarse, and quite brittle fibers. Moreover, pretreated OPEFB by modified pretreatment looks white in color with small, fine, and brittle fibers. This result means that sequential pretreatment using PA and AP solutions has an effect on the OPEFB structure, as shown in Fig. 1a and 1b. This is due to PA pretreatment was effectively damage and remove the lignin content on OPEFB, on the outer layer of the fiber (Azelee et al. 2014; Palamae et al. 2014). Therefore, the structure of non-modified pretreated OPEFB became more brittle. Otherwise, the AP pretreatment plays a role in solubilizing lignin/silica in order to expose the cellulose fiber. Therefore, overall pretreatment opened up places between lignocellulose structures by eliminating several materials in the fiber (Palamae et al. 2017).

Moreover, the assistance of ultrasound on the modified pretreatment, also affects the changes of OPEFB structure, compared to the non-modified pretreatment, as shown in Fig. 1b and 1c. This is due to the ultrasound produces physical effects to accelerates the disintegration and disruption of the OPEFB structure by surface erosion and destroying the compact structure of OPEFB (Bussemaker and Zhang 2013; Xu et al. 2017). Moreover, ultrasound has a role to break down the cell walls of the materials (Bussemaker and Zhang 2013). Therefore, changes in the OPEFB structure also occurred during the pretreatment assisted by ultrasound.

Moreover, sequential pretreatment also affects to the composition of OPEFB. The composition of untreated and pretreated OPEFB are depicted on the Table 1.

Table 1
Composition of untreated and pretreated OPEFB

Types of OPEFB	Content (%w/w)		
	Cellulose	Hemicellulose	Lignin
Untreated	49.29	30.28	20.43
Pretreated with non-modified pretreatment	55.66	27.29	17.05
Pretreated with modified pretreatment	68.40	18.33	13.27

Generally, OPEFB comprises of 43–65% cellulose, 17–33% hemicellulose, and 13–37% lignin based on dry weight basis (Khalil et al. 2012). Based on results, the composition of untreated OPEFB was consisted of 49.29% cellulose, 30.28% hemicellulose, and 20.43% lignin. Therefore, the composition of untreated OPEFB used in this study was in accordance with the previous reported ranges.

After subjected with sequential pretreatment, there were changes in the composition of OPEFB, including cellulose, hemicellulose, and lignin content. Pretreated OPEFB, either modified or non-modified, has higher cellulose content compared with the untreated OPEFB. The cellulose content on untreated OPEFB was 49.29%. Then, when OPEFB treated with non-modified pretreatment, cellulose content increased to

55.66%. Moreover, when treated with modified pretreatment, cellulose content on OPEFB increased to 68.40%. Therefore, the pretreatment has succeeded in increasing cellulose content in OPEFB. Moreover, modified pretreatment obtained a better increase in the cellulose content of OPEFB rather than non-modified pretreatment.

In the other side, lignin content in the pretreated OPEFB was lower than in the untreated OPEFB. The lignin content of untreated OPEFB was 20.43%. However, lignin content in OPEFB which treated with non-modified pretreatment was 17.05%. In addition, lignin content in OPEFB that treated with modified pretreatment was 13.27%. From these results, it could be seen that the lignin content in OPEFB was reduced by pretreatment. Therefore, the pretreatment has succeeded in removing lignin in OPEFB. On the other side, hemicellulose content in the pretreated OPEFB was also lower than in the untreated OPEFB. The untreated OPEFB has 30.28% of hemicellulose content. Whereas, the hemicellulose content in OPEFB pretreated with non-modified pretreatment was 27.29%. Meanwhile, the hemicellulose content in OPEFB pretreated with modified pretreatment was only 18.33%.

Changes in OPEFB composition were obtained due to the pretreatment process which performed on OPEFB. PA solution as the pretreatment agent is very selective in removing lignin, through several mechanisms including hydroxylation of aromatic rings, oxidative demethylation, opening of oxidative rings, and epoxidation (Song et al. 2013; Sundara 1998). Moreover, pretreatment with AP solution helps to remove hemicellulose content (Palamae et al. 2014).

Furthermore, the modified pretreatment assisted by ultrasound, has a significant effect on the composition of OPEFB. This is due to the ultrasound generates sonochemical and mechanoacoustic effects which affect the chemical and physical composition of biomass. The mechanoacoustic effect can change the surface area and crystalline structure of biomass, while the sonochemical effect can produce oxidizing radicals that lead to chemical attack on biomass (Lee et al. 2020). Moreover, ultrasound also works to help the break of homolytic bonds in lignin, therefore delignification process will be improved (Bussemaker and Zhang 2013). Based on overall results, this study suggested the modified pretreatment as the better pretreatment method for OPEFB.

Production of reducing sugar from modified-pretreated OPEFB during enzymatic hydrolysis

Production of reducing sugar through the enzymatic hydrolysis using modified-pretreated OPEFB was performed and investigated in this study. The enzymatic hydrolysis were carried out for 48 hours in different concentration of enzyme. The enzyme concentration used in this study were 1.25, 2.5, and 5 g/L. The production of reducing sugar from modified-pretreated OPEFB during enzymatic hydrolysis in different concentration of enzyme are represented in the Fig. 2.

Based on overall results, production of reducing sugar increases gradually along the hydrolysis time in all concentration of enzyme. On the enzyme concentration of 1.25 g/L, during 0–6 hours, sugar was produced exponentially, almost up to 5 g/L. After 6 hours, sugar concentration was still increase linearly until 48 hours, with the final sugar concentration of 8.49 g/L. Besides, on the enzyme concentration of 2.5 g/L, sugar was also significantly produced during 0–6 hours, up to 9.14 g/L. However, after 6 hours, the

increase of sugar concentration was not significant until 48 hours, with the final sugar concentration of 11.06 g/L. Furthermore, on the enzyme concentration of 5 g/L, sugar was also rapidly produced during 0–2 hours, up to 16.61 g/L. Along 2–6 hours, sugar production was started to be stationary, up to 17.98 g/L. However, after 6 hours until 48 hours, sugar production was very insignificant, with the final sugar concentration of 19.16 g/L.

Effect of hydrolysis time on the production of reducing sugar

Enzymatic hydrolysis were performed for 48 hours. During hydrolysis, sugar were analyzed at several points of time in order to obtain the sugar concentration in each point of the time. The sugar were analyzed at 30 and 45 minutes and at 1, 2, 4, 6, 24, 30, and 48 hours. Overall results are represented on the Fig. 2.

Based on Fig. 2, it can be seen that the high increase of sugar production were achieved under 6 hours of hydrolysis time, at all concentration of enzyme. On the enzyme concentration of 1.25 g/L, the highest increase of sugar concentration was occurred during 30 to 45 minutes, as much as 1.095 g/L, from 0.592 to 1.687 g/L of sugar concentration. However, on the enzyme concentration of 2.5 and 5 g/L, the highest increase of sugar concentration were achieved during the initial 30 minutes of the hydrolysis time. On the enzyme concentration of 2.5 g/L, concentration of sugar obtained in 30 minutes was 5.01 g/L. Otherwise, on the enzyme concentration of 5 g/L, concentration of sugar produced in 30 minutes was 10.6 g/L.

Furthermore, during 6 to 48 hours of hydrolysis time, the increase of sugar concentration were not significant, in all concentration of enzyme. On the enzyme concentration of 1.25 g/L, the increase of sugar concentration only reached 1.79-fold with an 8-fold increase in hydrolysis time. However, on the enzyme concentration of 2.5 g/L, the increase of sugar concentration was only 1.21-fold. Furthermore, on the enzyme concentration of 5 g/L, the increase of sugar concentration was 1.09-fold. Based on overall results, it can be concluded that hydrolysis process after 6 hours were no longer effective, since the sugar production were insignificant. Thus, this study recommends an effective hydrolysis time of 6 hours for sugar production. However, decreased or constant production of reducing sugar after 6 hours can be caused by the accumulation of cellobiose which may induce severe feedback inhibition of the enzyme activities, thus obtaining low hydrolysis yield (Sarkar and Aikat 2014). Besides, this also can be due to the presence of binding between enzymes and residual lignin contained in biomass (Menegol et al. 2014).

This phenomenon can be explained that in general, production of sugar in the hydrolysis process can be divided into two periods. The first period is associated with the fast release of soluble sugars and the second period is associated with the declining rate of sugar production (Ortega et al. 2001). Based on this principle, in this study, the first period were happen during 0–6 hours, otherwise the second period were happen after 6 hours of hydrolysis until the reaction ends at 48 hours. This results were also similar with the previous study that exhibits fast reaction rate of hydrolysis during the first 5 hours and further increase up to 6 hours (Singh et al. 2019).

Furthermore, the increase of sugar concentration during 6 to 48 hours were decreased as the increasing of enzyme concentration. This might be happen due to the increase of enzyme concentration will

increase the initial conversion of cellulose and increase the initial sugar production significantly during 0 to 6 hours. Therefore, with the constant amount of substrate, the conversion of cellulose and sugar production after 6 hours will be reduced as the increasing of enzyme concentration.

Effect of enzyme concentration on the production of reducing sugar and hydrolysis yield

The modified-pretreated OPEFB were enzymatically hydrolysed by cellulase enzyme with different concentration of enzyme. The enzyme concentration used were 1.25, 2.5, and 5 g/L based on the working volume of hydrolysis process. Hydrolysis were performed at 50°C for 48 hours. Thus, final reducing sugar production and hydrolysis yield at 48 hours will be studied in this section. Moreover, as mentioned in the previous section, the effective time for hydrolysis process was 6 hours. Thus, the effect of different concentration of enzyme to the sugar concentration produced and hydrolysis yield will also be evaluated in 6 hours. Production of reducing sugar and hydrolysis yield at 6 and 48 hours are shown in the Fig. 3.

Based on overall results, concentration of reducing sugar produced and hydrolysis yield were increased as the increasing concentration of enzyme, both at 6 hours and 48 hours of hydrolysis time. This is due to the increase in enzyme concentration will increase the availability of the active site on the enzyme, thus more peptide bonds in cellulose will be broken by the enzyme (Kurozawa et al. 2008). Therefore, it will increase the amount of reducing sugar produced and hydrolysis yield, since cellulose and hemicellulose were converted into reducing sugar.

According to Fig. 3a, at the enzyme concentration of 1.25 g/L, the concentration of reducing sugar achieved at 6 hours was 4.73 g/L and at 48 hours was 8.49 g/L. Moreover, when the enzyme concentration increased at 2.5 g/L, the concentration of reducing sugar produced were 9.14 and 11.06 g/L, at 6 hours and 48 hours of hydrolysis time, respectively. Furthermore, at 5 g/L of enzyme concentration, 17.98 g/L of reducing sugar was produced at 6 hours and 19.16 g/L of reducing sugar was obtained at 48 hours.

From these results, at 48 hours of hydrolysis time, it can be seen that when the enzyme concentration increased by 2 times at 2.5 g/L, the final sugar concentration obtained was increase 1.3 times from 8.49 to 11.06 g/L. Furthermore, when the enzyme concentration increased by 4 times from the initial enzyme concentration, at 5 g/L, the final sugar concentration attained was increase 2.25 times at 19.16 g/L. In addition, at the hydrolysis time of 6 hours, a 2-fold increase in the enzyme concentration from 1.25 to 2.5 g/L, increased the sugar concentration by 1.93 times from 4.73 g/L to 9.14 g/L. Furthermore, a 4-fold increase in the enzyme concentration from 1.25 to 5 g/L, obtaining a 3.8-fold increase in sugar concentration to 17.98 g/L.

These results were similar with the other study which exhibits an increase of sugar production along with the increase of cellulase amount used in the hydrolysis of formiline-pretreated OPEFB. When the cellulase loading were increased 2 and 3 times, sugar concentration increased significantly. However, when the cellulase loading was increased 4 times, an increase on sugar concentration was also found, although only slightly (Cui et al. 2014). Other than that, related results were also found from the OPEFB hydrolysis

pretreated with dilute acid, obtaining the best experimental results at the highest observed enzyme loading (Gonzales et al. 2019).

The same trend was also obtained on the hydrolysis yield. Since hydrolysis yield is depend on the concentration of reducing sugar produced, therefore the similar trend was also achieved, as seen in Fig. 3b. At the enzyme concentration of 1.25 g/L, the hydrolysis yield of 24.52% and 44.04% were achieved at 6 and 48 hours, respectively. Other than that, the hydrolysis yield obtained at the enzyme concentration of 2.5 g/L were 47.43 and 57.38%, at 6 hours and 48 hours, respectively. Furthermore, significant hydrolysis yield was exhibited when the enzyme concentration of 5 g/L was applied. Hydrolysis yield of 93.31% and 99.4% were obtained at 6 and 48 hours, respectively.

Based on overall results, the highest reducing sugar concentration and hydrolysis yield were achieved by the highest enzyme concentration at 5 g/L, both at 6 hours and 48 hours of hydrolysis time. Therefore, this study suggests 5 g/L as the optimum enzyme concentration used for hydrolysis process in order to produce reducing sugar from the pretreated OPEFB.

Effect of different pretreatment on the production of reducing sugar and hydrolysis yield

Effect of different pretreatment method on the production of reducing sugar and hydrolysis yield from OPEFB were also evaluated in this study. The concentration of reducing sugar produced and hydrolysis yield from OPEFB which treated by different pretreatment methods are depicted in the Fig. 4.

The concentration of reducing sugar and hydrolysis yield presented were obtained under the optimum condition of hydrolysis process, at the enzyme concentration of 5 g/L and 6 hours of hydrolysis time. As seen in Fig. 4a, concentration of reducing sugar produced by OPEFB which treated by modified pretreatment was higher than the concentration of reducing sugar produced by OPEFB that treated by non-modified pretreatment. The concentration of reducing sugar produced by OPEFB treated by modified pretreatment was 17.89 g/L. Meanwhile, the concentration of reducing sugar produced by OPEFB treated by non-modified pretreatment was 16.61 g/L. Based on these results, there was an increase in sugar concentration by 8.25% in the hydrolysis of OPEFB which treated by modified pretreatment compared to the non-modified pretreatment.

The increase of reducing sugar concentration on the modified-pretreated OPEFB may occurred due to the synergistic effect of sequential peracetic acid-alkaline peroxide solution and ultrasound pretreatment. The mechanism of modified and non-modified pretreatment followed by enzymatic hydrolysis of OPEFB is presented in Fig. 5.

During the modified pretreatment process, ultrasound produces mechanoacoustic effect by generating the pressure wave, which leading to the formation and development of microbubbles (Teh et al. 2017). As the time microbubbles achieve the critical size, cavitation of microbubbles will occurs creating extreme pressure to the surroundings. Microbubbles will then collapse and a microjet, a strong stream of liquid, will be directed to the biomass surface (Teh et al. 2015). The high pressure liquid stream would then lead to the disintegration of surface layer of the biomass and perforations of cell wall thus increasing the

surface area of the biomass (Ong et al. 2019). Increased surface area would increase the contact between pretreatment reagent and biomass in the pretreatment process, thus increasing the removal of lignin content in the biomass.

Moreover, using peracetic acid solution as the pretreatment reagent, facilitating the alteration of lignin structure (Mosier et al. 2005) by solubilizing the acid insoluble lignin. Furthermore, hydrogen peroxide used in the pretreatment reagent produces hydroxyl radicals ($\text{HO}\cdot$) and superoxide anion radicals ($\text{O}_2^{\cdot-}$), which cause the oxidation of lignin structures (Sun et al. 2000). In the other hand, alkaline solution also enhanced lignin solubilization by improving the degradation of ester bonds and carbon-carbon bonds in lignin molecules by hydroxyl anions (OH^-) (Moodley and Kana 2017). As a result, modified pretreatment assisted by ultrasound resulting higher reduction of lignin rather than non-modified pretreatment. Since the lignin content decreased and cellulose content increased in modified-pretreated OPEFB, as seen in Table 1, consequently an increase of reducing sugar concentration was achieved in this study. The similar results were also observed by other researcher undergoing combination of ultrasonication and deep eutectic solvent (DES) pretreatment of oil palm frond obtaining lower lignin content and retaining high hemicellulose and cellulose content, thus releasing high glucose yield in enzymatic hydrolysis process (Ong et al. 2020).

Moreover, the increase of reducing sugar concentration also can be attributed to the higher surface area achieved due to the mechanoacoustic effects of ultrasound which improve the mass transfer and enhance the accessibility of enzymes into the biomass in enzymatic hydrolysis process, as illustrated in Fig. 5. Furthermore, the alkaline solution used in the pretreatment process also caused biomass fibers swelling, which endorsed enzymes to infiltrate deeper into the biomass (Loow et al. 2016). Therefore, as the subsequent process, performance of enzymatic hydrolysis was enhanced due to the synergistic effect of sequential peracetic acid-alkaline peroxide solution and ultrasound, represented by the increase in sugar concentration. The previous study also demonstrated a similar result performing ultrasound-assisted alkaline pretreatment on rice straw to increase the digestibility for enzymatic hydrolysis (Wu et al. 2017). This study attributed the increase in sugar concentration to the increased accessibility of cellulose resulting from a higher specific surface area after alkaline pretreatment. In addition, the study also revealed that the synergistic effect of ultrasound and alkaline solution pretreatment was able to produce higher concentration of reducing sugar.

However, in terms of hydrolysis yield, there was only a slight increase between biomass pretreated with modified and non-modified pretreatment, as seen in Fig. 4b. Another study also demonstrated slight improvement of glucose yield on enzymatic hydrolysis of food waste pretreated by ultrasound. The glucose yield obtained on pretreated food waste around 10% higher rather than untreated food waste (Li et al. 2019). However, the study revealed that applying ultrasonic pretreatment may improve the hydrolysis rate, since the time required to achieve high yield in the study being halved compared to the untreated food waste. The present study, exhibiting the similar results with the previous study. In the present study, the sugar yield obtained from modified-pretreated OPEFB (assisted by ultrasound) was 93.31% in 6 hours. However, on the non-modified OPEFB (without the assistance of ultrasound), the

comparable yield value was achieved within 30 hours. The results implied that the time required by modified-pretreated OPEFB in this study only a fifth compared to the non-modified OPEFB.

Furthermore, several enzymatic hydrolysis of various lignocellulose biomass were also enhanced by performing the ultrasound pretreatment. Improvement of glucose yield also attained from oil palm frond pretreated by NaOH-aqueous deep eutectic solvent (DES) assisted by ultrasound. The glucose yield achieved after 72 hours of enzymatic hydrolysis was 90.02% (Ong et al. 2020). Furthermore, it was also found that combination of ultrasonic and dilute aqueous ammonia pretreatment subjected to several biomass can improve the accessibility of cellulose in biomass and also improve the sugar yield on enzymatic hydrolysis. The largest sugar yield obtained from corncob, corn stover, and sorghum stalk were 80.6%, 66.2%, and 56.9%, respectively (Xu et al. 2017). Compared with the present study, the yield of 93.31% has been achieved on modified-pretreated OPEFB in 6 hours of enzymatic hydrolysis. Therefore, the modified pretreatment performing sequential peracetic acid – alkaline peroxide combined with ultrasound carried out in the present study, has considerable results compared by the previous results from other researchers.

Conclusions

Production of reducing sugar from enzymatic hydrolysis of OPEFB had been improved by performing modified pretreatment using sequential PA – AP solution assisted by ultrasound. The highest reducing sugar production of 19.16 g/L was obtained using 5 g/L of cellulase enzyme concentration at 48 hours. However, the effective time of hydrolysis was reached at 6 hours, since the sugar production at all concentration of enzyme after 6 hours was insignificant. Therefore, the reducing sugar production obtained at 5 g/L of enzyme concentration for 6 hours was 17.98 g/L with the hydrolysis yield of 93.31%. Based on results, an increase in sugar concentration by 8.25% has been achieved in the hydrolysis of OPEFB pretreated by modified pretreatment method compared with the OPEFB pretreated by non-modified pretreatment method.

List Of Abbreviations

OPEFB	: oil palm empty fruit bunch
PA	: peracetic acid
AP	: alkaline peroxide
CH ₃ COOH	: acetic acid
H ₂ O ₂	: hydrogen peroxide
NaOH	: sodium hydroxide
NREL	: National Renewable Energy Laboratory

HO[•] : hydroxyl radicals

O₂^{•-} : superoxide anion radicals

OH⁻ : hydroxyl anions

DES : deep eutectic solvent

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

DNP was responsible for the overall experimental work, data analysis, and manuscript writing. MSP has a role in manuscript editing. MY has a role to provide the resources for hydrolysis experiment, including materials, equipment, and laboratory facility. TSU and MS have a role as supervisor to review the experimental results and manuscript writing and revisions. HH has a role as corresponding author.

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Figures

Figure 1

Physical appearance of OPEFB: a untreated; b pretreated by non-modified pretreatment; c pretreated by modified pretreatment

Figure 2

Production of reducing sugar from modified-pretreated OPEFB during hydrolysis in different concentration of enzyme: (a) 1.25 g/L; (b) 2.5 g/L; (c) 5 g/L

Figure 3

Concentration of reducing sugar and b hydrolysis yield in different concentration of enzyme

Figure 4

a Concentration of reducing sugar produced and b hydrolysis yield under different pretreatment method

Figure 5

Mechanism of modified and non-modified pretreatment followed by enzymatic hydrolysis

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