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Biobutanol production from cocoa pod husk through a sequential green method: Depectination, delignification, enzymatic hydrolysis, and extractive fermentation

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ABSTRACT

The effect of various treatments on the production of reducing sugar and biobutanol from cocoa pod husk (CPH) through depectination, delignification, enzymatic hydrolysis, and fermentation was studied to obtain the best strategy for sequential processes to valorize CPH to biobutanol. Surfactants of Tween 80, Polyethylene Glycol (PEG) 6000, and Sodium Dodecyl Sulfate (SDS) was added to enhance the performance of enzymatic hydrolysis, and L-cysteine addition to support fermentation by *Clostridium saccharoperbutylacetonicum N1–4*. The optimum activity of the cellulase was at 0.25 FPU/g. Depectination, microwave-assisted delignification, and 2.5 g/L PEG 6000 addition sequence produced the highest sugar concentration of 9.24 g/L. The highest butanol and acetone-butanol-ethanol (ABE) concentrations of 20.4 g/L and 54.4 g/L, respectively, were obtained using immobilized cells and L-cysteine supplementation. ABE production by extractive fermentation was enhanced using immobilized cells and L-cysteine supplementation.

1. Introduction

The growing global demand for cocoa increases the cocoa pod husk (CPH) generation worldwide. The average husk part reaching 76 % of cocoa fruit is potential for renewable feedstock, which has not been fully utilized yet. CPH contains hemicellulose 8.7–12.8 %, cellulose 19.7–26.1 %, lignin 14–28 %, and 6–12.6 % pectin (Lu et al., 2018). The holocellulose containing cellulose and hemicellulose (xylan & arabinoxylan) can be hydrolyzed to produce reducing sugar for fermentation substrate while lignin inhibits the hydrolysis process. CPH hydrolysate contains feedstock sugar which is fermentable to produce biobutanol as a gasoline substitute (Campos-Vega et al., 2018). Production of biobutanol is assisted by butanol producing strains such as *Clostridium acetobutylicum* YM1, *Clostridium beijerinckii,* and *Clostridium saccharoperbutylacetonicum* N1–4 which convert mono and oligosaccharides of CPH hydrolyzate into ABE (Acetone-Butanol-Ethanol) (Ozturk et al., 2022). Depectination and delignification ease the access of enzyme to the holocellulose that increases the reducing sugar yield. The pectin extraction opens the blockage of pectin for the enzyme to degrade holocellulose while lignin is a phenolic compound strengthening the cell wall that inhibits holocellulose conversion. In addition, pectin and lignin can be utilized to produce valuable products. Pectin is used as a gelling agent, stabilizer, thickener for food, wound healing, cholesterol reducer, and many more. Lignin could be used for boiler fuel, adhesives, and cement additives (Grossman and Wilfred, 2019).

Pectin is extracted by heating extraction (HE) using acid, which is a widely used method. Citric acid is an organic acid which is effectively extracting pectin with high yield and physicochemical quality. It is natural and safe for food and thus is more interesting than other strong acids such as nitric, chloric, or sulphuric acid for commercial pectin

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extraction. This is the most advantageous extracting agent both economically and environmentally (Lee and Choo, 2020). The weaknesses of the HE method are lower surface tension and longer extraction time. Due to those weaknesses, it requires additional extraction method such as ultrasound-assisted extraction (UAE) method (Kazemi et al., 2020). After pectin is extracted, lignin is reduced to enhance the enzyme accessibility and polysaccharide digestibility by destructing the lignin bound. Delignification with *Microwave-Assisted Alkaline* (MAA) is an effective method to reduce the lignin content of CPH. Microwave facilitates indirect energy absorption by the molecules so that it fastens the breaking of lignin structure by the molecules collision induced from dielectric polarization (Muharja et al., 2021). The intensified pretreatments of depectination using ultrasound and delignification using microwave are expected to release pectin and lignin from CPH effectively in shorter time.

Enzymatic hydrolysis with surfactant addition is recently used to increase sugar yield and reduce the enzyme loading in the production of sugar from pretreated biomass. The surfactant reduces the surface tension, the hydrophobic part interacts with the non-aqueous phase, and the hydrophilic part interacts with the aqueous phase. *Hydrophilic–lipophilic balance* (HLB) of the surfactant is determined by the chemical characteristic of its hydrophilic and hydrophobic groups. High HLB surfactants are good additives for enhancing degradation of lignin and hemicellulose. For example, Tween-80 (HLB, 15.0), and PEG 6000 (HLB, 19.15) with high HLB are potential for catalysts during delignification and enzymatic hydrolysis (Muharja et al., 2019). Therefore, these studies used those surfactants to obtain the optimum results of reducing sugar production.

The study of biobutanol from CPH is very limited despite its promising future for gasoline and chemical substitutes. Butanol is produced using fermentation by bacteria converting mono and oligosaccharide into Acetone-Butanol-Ethanol (ABE) (Darmayanti et al., 2021; Ozturk et al., 2021). The toxicity effect of ABE in the fermentation is reduced by integrated separation. Extractive fermentation with immobilized cells is an effective method to reduce and protect the cells from the produced solvent while maintaining cells viability (Darmayanti et al., 2018). Moreover, the addition of L-cysteine as a reducing agent enhances the cell performance to produce butanol with a higher concentration. It increases NADH generation which gives higher enzyme activity for butanol production (Chandgude et al., 2021). The extractive fermentation of CPH hydrolysate using immobilized cells with L-cysteine supplementation has not been investigated.

This study aim to utilize CPH for butanol production through a series of processes: ultrasonic-assisted pectin extraction, microwave-assisted delignification, enzymatic hydrolysis with surfactant addition, and extractive fermentation using immobilized cells with reducing agent supplementation. This study examined the effect of the pectin extraction and delignification of CPH, to the result of hydrolysis process which has not been investigated so far. This research is the first to study enzymatic CPH hydrolysis with surfactant addition and the enhanced fermentation with immobilized cells and integrated extraction, supported with reducing agent. The optimum condition for each process of biobutanol production from CPH was studied.

2. Materials and methods

2.1. Pectin extraction with solvent extraction method

CPH was collected from the cocoa plantation in Jember, Jawa Timur, Indonesia. CPH was dried under the sun for 72 h and the moisture content was reduced to approximately 10 % by sun drying. Then, it was ground and sieved to 100 mesh size. Pectin was extracted with 7 % citric acid (Merck, Singapore) based on the best condition of previous research (Muñoz-Almagro et al., 2019). The ratio of solid to liquid was 1:20 (w/v). Heating extraction was conducted for particle sizes of 60, 80, and 100 mesh. The temperature variations of 25 °C, 50 °C, and 75 °C, and solid loading variation of 2.5 g, 5 g, and 7.5 g during 30 min, 60 min, and 90 min, and for ultrasonic-assisted extraction solvent addition of 100 mL, 150 mL, and 200 mL along 10 min, 20 min, 30 min, and 40 min. The results of the pectin extract were filtered with a filter cloth while the extract was still hot. The filtrate was cooled and precipitated by adding ethanol 96 % (Merck, Germany), then stirred until the deposit was formed, and then put in a dark place for 24 h. The deposit was separated from the solution using a filter cloth. Then it was washed with 100 mL ethanol and dried in the air-oven at 50 °C for 24 h. The dried pectin samples were kept for further analysis.

2.2. Microwave-Assisted Alkali (MAA) delignification

Delignification was conducted based on the optimal results obtained from the previous study (Muharja et al., 2021). The MAA process was run under the mild condition by mixing 5 g of CPH 80 mesh and 3 % NaOH (Merck) solution with a sample to a solvent ratio of 0.035 g/L in the beaker glass. Then, the sample was heated in a modified domestic microwave oven (100 W) for 2.5 min irradiation time. After delignification, the solid was filtered and dried in the oven at 45 °C for a day.

2.3. Enzymatic hydrolysis

Enzymatic hydrolysis was conducted in a 250 mL flask. In the flask, 5 g of pretreated CPH was mixed with cellulase enzyme Viscozyme Cassava CL (Novozymes A/S, Denmark) of 0.1, 0.25, 0.5, 0.75, 1, 5, 10, and 15 FPU/g. The 4.8 pH citrate buffer of 0.05 M was added at the solid to liquid ratio of 1:10 (w/v). The flask was tightly sealed and incubated at 60 °C 125 RPM in an incubator shaker (B-ONE SIC 50 L, China). Hydrolysis was run up to 48 h with a sampling interval of 6 h. The sample with optimum enzyme loading was selected for the enzymatic hydrolysis with pro-analysis grades surfactant of Tween 80 (Merck, France), Polyethylene Glycol 6000 (PEG 6000) (Merck, Germany), Sodium Dodecyl Sulfate (SDS) (Merck, Germany) with the concentration of 2.5 g/L and 5 g/L.

2.4. Microorganism inoculation

Clostridium saccharoperbutylacetonicum N1–4 (ATCC 13564) was obtained from the Laboratory of Microbial Technology, Department of Biosciences and Biotechnology, Kyushu University. *C. saccharoperbutylacetonicum* N1–4 sand stock was refreshed into 9 mL potato glucose (PG) media 10 % v/v inoculum. The PG was then heat-shocked in 100 °C water for 1 min. Then the refresh culture was incubated at 30 °C for 24 h. The refresh culture was then inoculated into tryptone-yeast extract-acetate (TYA) media containing tryptone bacto 6 g/L, yeast extract 2 g/L, CH_3COONH_4 3 g/L, $MgSO_4.7H_2O$ 0.3 g/L, $FeSO_4.7H_2O$ 0.01 g/L, and KH_2PO_4 0.5 g/L. The preculture was TYA containing 20 g/L glucose and 10 % v/v refresh culture. The preculture was incubated at 30 °C for 15 h in an anaerobic condition.

2.5. Extractive fed-batch fermentation

The main culture contained 60 mL TYA media with 50 g/L glucose (Merck, Germany), 10 % v/v inoculum from preculture, $CaCO_3$ 3 g/L, and 150 mL palm oil as extractant. The mixture was then sparged with nitrogen gas for 5 min. The fermentation was run at 30 °C 125 rpm with 2 mL CPH hydrolysate (containing 9 g/L sugar) addition at 24 h, 36 h, 48 h, and 72 h. The fermentation was ended at 96 h. All the experiment was performed and examined in triplicate.

Immobilized cells beads were prepared by making sodium alginate gel (3 % w/v) containing NaCl (0.85 % w/v) and preculture media of 10 % v/v. The gel mixture was then dropped in $CaCl_2$ solution (3 % w/v) to form calcium alginate beads. The beads were filtered and used for fermentation. For extractive fermentation using immobilized cells, the working volume of TYA was 60 mL without preculture, 60 mL cells

beads, and 150 mL palm oil extractant. L-cysteine (Merck) 0.5 g/L was added in TYA media.

2.6. Analytical methods

Reducing sugars from enzymatic hydrolysis was analyzed by the dinitrosalicylic acid (DNS) (Sigma-Aldrich) using Spectrophotometry UV–Vis (Vernier Go Direct GDX-SVISPL, China) at 540 nm as previously reported (Muharja et al., 2017). Butanol, acetone, and ethanol were measured in the media using gas chromatography (Trace 1300, ThermoScientific, Italy) with a flame ionization detector and a TR Wax column. The column was operated at 50–190 °C. Nitrogen was used as a carrier gas. Inlet was set with the split mode at 50 °C, pressure 14.16 psi, total flow 10.1 mL/min, and split ratio 50:1. The oven was adjusted at 50 °C on the initial temperature, then ramped 10 °C/min into 170 °C. The front inlet detector was set at 250 °C. The hydrogen, air, and nitrogen flow were set at 40, 450, and 3.7, respectively.

2.7. Statistical analysis and calculation

Analysis of Variance (ANOVA) was used to determine the significance of sequel treatment and surfactant addition in hydrolysis using Minitab 16 (Minitab Inc., Institut Teknologi Sepuluh Nopember, Indonesia).

The yield of the pectin was calculated by Eq. (1) (Muñoz-Almagro et al., 2019).

$$Pectin\ yield\ (\%) = \frac{dry\ pectin\ mass}{dry\ CPH\ mass} \times 100\% \quad (1)$$

The total hydrolysis was defined as following Eq. (2) (Muharja et al., 2018).

$$Total\ hydrolysis\ (\%) = \frac{total\ reducing\ sugar\ (g)}{initial\ cellulose\ and\ hemicellulose(g)} \times 100\% \quad (2)$$

The distribution coefficient (Kd) and the percentage of extraction efficiency (E) were calculated using the following Eqs. (3) and (4) (Khedkar et al., 2020).

$$Kd = \frac{C_{org}}{C_{aq}} \quad (3)$$

$$E\ (\%) = \frac{C_{org}}{C_{feed}} \times 100\% \quad (4)$$

where Kd is the distribution coefficient, E is the percentage of extraction efficiency, and C_{org}, C_{aq}, and C_{feed} are the concentrations of extraction, media, and extractant + media, respectively.

3. Results and discussion

3.1. Pectin extraction

3.1.1. Conventional heating extraction

Fig. 1 shows the effect of various operating conditions of conventional heating extraction on pectin yield. The effect of CPH size is demonstrated in Fig. 1a. The CPH size had a significant effect on the pectin yield ($P < 0.05$). The yield increased as the particle size decreased. However, further reduction of the particle size will reduce the pectin yield. The highest yield was obtained when the particle size was 80 mesh, then decreased after that optimum point. This could be

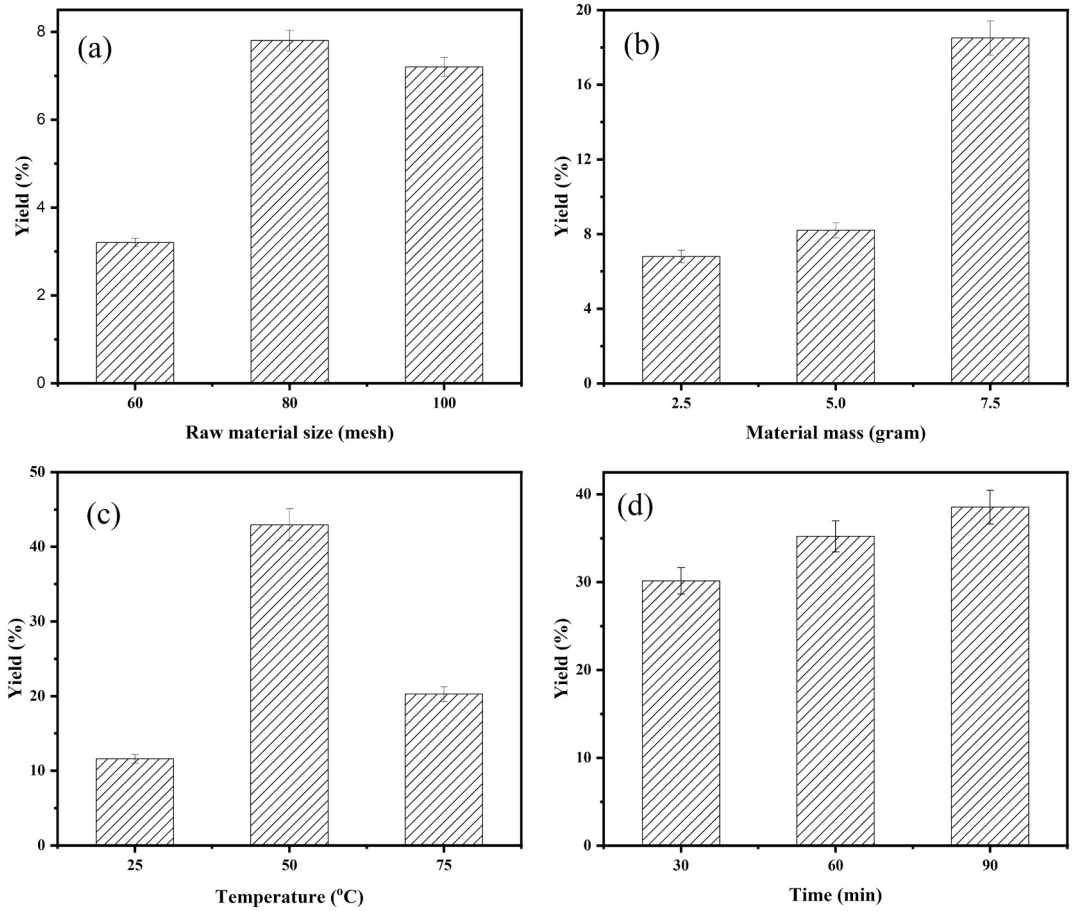

Fig. 1. Pectin yield using conventional extraction heating with the variation of raw material size (a), material mass (b), temperature (c), and time (d).

due to the retention of the filtrate. The filtrate containing the polysaccharide pectin in smaller size materials will absorb a lot of solution to form a gel (Freitas et al., 2020). The amount of extract obtained depends on the mass of the CPH used. The yield will be higher with the addition of CPH, as shown in Fig. 1b. The largest yield of 18.5 % was produced when the mass of the material was 7.5 g, while the smallest yield of 6.8 % was obtained when the mass of the material was 2.5 g.

Extraction using solvents is a type of process that involves mass transfer. Mass transfer can be affected by heat transfer. It has been shown in Fig. 1c that the rate of heat transfer increased the extraction because the dissolution process occurred more rapidly at higher temperatures (Mao et al., 2021). The yield of pectin increased as increasing temperature but decreased after reaching its optimum point. The largest pectin yield of 42.93 % was attained at a temperature of 50 °C. Increasing the temperature significantly increases the yield of pectin. As reported by Ma et al. (2019), an increase in pectin yield of 3.8 % occurs for every increase in temperature of 10 °C. The heated acid helped dissolve pectin and other pectin components that are stuck in the cell wall (protopectin), thereby increasing the yield of pectin. Acid at low temperature is not sufficient to hydrolyze protopectin (the insoluble form of pectin), so the yield of pectin obtained is lower than using high temperature. However, in this study, at higher temperatures, the yield was reduced by ~53 % due to the degradation of the dissolved pectin. Another study emphasized that the extraction of pectin at higher temperatures could destruct the molecular structure of pectin and decrease the viscosity and the binding affinity of the galacturonic acid (Chen et al., 2020).

The yield of pectin obtained was directly correlated with the increase in extraction time. Yield increased as heating time increased (Fig. 1d). This is because the contact time between the solvent and solute is getting longer so that more protopectin is hydrolyzed into pectin (Adetunji et al., 2017). However, some studies reported that further heating would reduce the degree of methylation, which could reduce the capacity of pectin as a thickener in the food industry (Belkheiri et al., 2021). In this study, the highest pectin yield of 38.53 % was obtained at 90 min.

3.1.2. *Ultrasound-assisted extraction*

Fig. 2 shows the effect of various operating conditions of ultrasound-assisted extraction on pectin yield. Particle size had a minor effect on the extraction of CPH using UAE, as shown in Fig. 2a. The smaller the size of the material, the higher the yield of pectin produced. This phenomenon may be due to the contact surface area of the material getting bigger so that it allows the extraction process to occur more evenly (Adetunji et al., 2017). However, in the various particle sizes used, the particle size did not significantly affect the pectin yield ($P > 0.05$). Lu et al. (2018) review that the pectin extraction from CPH can be economically feasible by decreasing particle size and will give a profit on a commercial scale production. The highest yield of 6 % was obtained at the particle size of 60 mesh.

The ultrasonication time is one of the important process parameters that significantly affect the pectin yield. The effect of time on the extraction of pectin from CPH waste is shown in Fig. 2b. From Fig. 2b, there was a gradual increase in pectin yield up to 30 min and then decreased with increasing extraction time. This phenomenon may be due to the swelling and hydration of the plant material, which can be accelerated by the cavitation effect of ultrasonic waves during the extraction period (Khadhraoui et al., 2021). The breakdown of the structure and decomposition of the plant cell wall by the UAE can support the process of entering solvents into cells. This process causes an increase in the diffusion of pectin content from the biomass to the solvent, thereby increasing the extraction performance. However, the heating effect and exposure to ultrasonic treatment for a longer extraction time lead to structural damage and decomposition of pectin, which will reduce the extraction yield. Besides, long time and low concentration are not economically feasible conditions (Martinez-Solano et al., 2021).

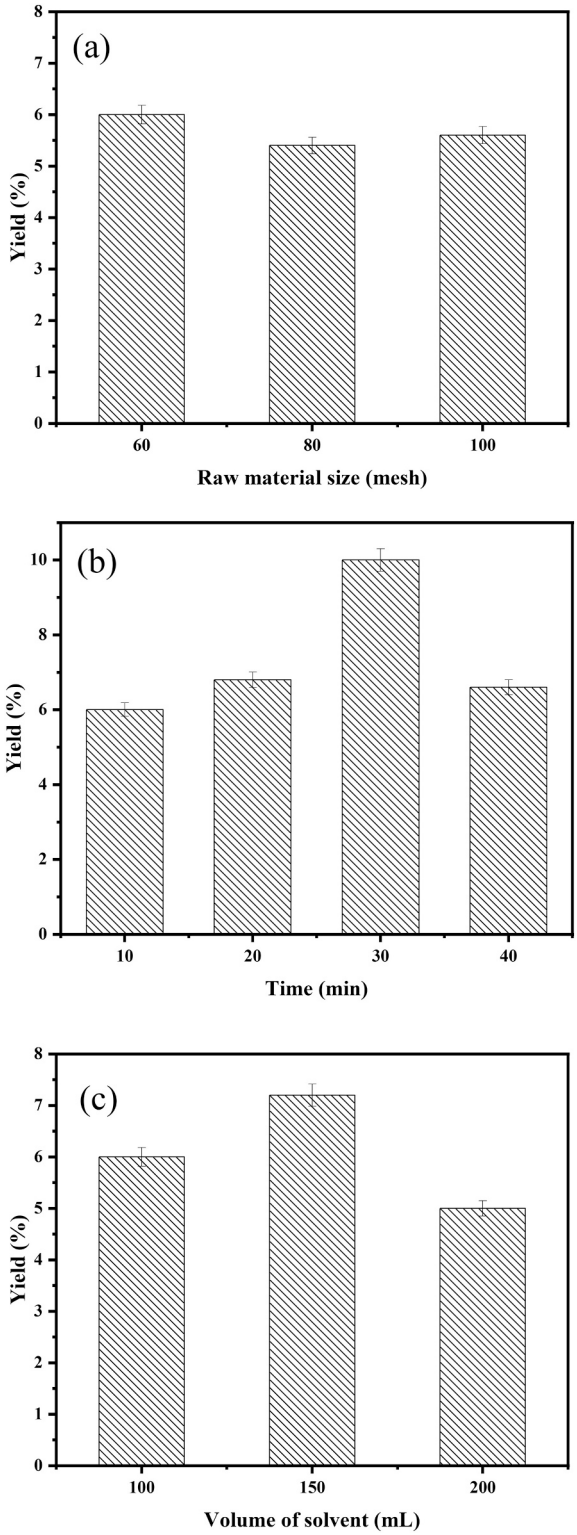

Fig. 2. Pectin yield using ultrasound-assisted extraction with the variation of size of raw material (a), time (b), and volume of solvent (c).

The selection of citric acid as a solvent was due to its environmentally friendly nature and its effectiveness compared to other mineral acids in producing pectin and maintaining its physicochemical properties (Mendes et al., 2019). To improve the extraction yield of the target analyte, a large-capacity solvent was used to effectively dissolve the compound from the material. Therefore, it was necessary to test the effect of solvent volume on the extraction results. Fig. 2c depicts the

effect of solvent volume on pectin yield using UAE. Increasing the volume, in the initial stage, increased the yield but then decreased the yield after reaching an optimum value (Fig. 2c). The highest yield was obtained when the citric acid volume was 150 mL. The increase in solvent volume decreases the concentration so that the mass transfer resistance decreases, resulting in an increase in pectin yield (Khadhraoui et al., 2021). The mass transfer occurred during the adsorption and penetration of the acid to CPH particles, then inversely the extracted pectin was transferred from CPH particles to the bulk solution. The increase in solvent indicates a decrease in the ratio from solid to solvent. On the other hand, the concentration gradient as the driving force of mass transfer increases as the solvent volume increases (Adetunji et al., 2017). The rise in solvent volume from 100 mL to 150 mL in Fig. 2c causes a decrease in the mass transfer resistance, yielding an increase in pectin recovery. Further increase in volume from 150 mL to 200 mL causes the concentration gradient as the driving force of mass transfer to decrease, resulting in a decrease in the mass transfer rate and yield. Ultrasonic waves rapidly pass from the solvent system to the biomass and increase the efficiency of pectin yield. However, prolonged exposure reduces the supply of ultrasonic energy density and negatively impacts the extraction yield. The amount of solvent above the optimum point causes cavitation bubbles to occur, which will reduce the pectin yield.

3.2. Effect of cellulase enzyme dosage on hydrolysis

Enzymatic hydrolysis is one of the important processes for producing monosaccharides or oligosaccharides from polysaccharides. The results of hydrolysis are highly dependent on the solid content and the dose of the enzyme used. High biomass loading can suppress hydrolysis results due to the decrease in the cellulose conversion and sugar yield. Fig. 3 presents reducing sugar concentration for various doses of enzyme. The hydrolysis time has a significant effect (P-value = 0.034) on the sugar concentration, as shown in Fig. 3a. The yield of reducing sugar increases with the length of hydrolysis time, but then decreases after the optimum condition. The sugar yield during the enzymatic hydrolysis for the first 12 h consistently increased. In the time course of 6 h, it shows that the higher the dose of the enzyme used, the higher the rate of reducing sugar formation. Hydrolysis in the range of 12 to 48 h shows a constant sugar recovery (no significant increase), and the process occurs more slowly. The concentration did not increase significantly since the glucose available in the substrate had been hydrolyzed by the enzyme so that only a small amount of substrate remained (Chen et al., 2018).

The dose of enzymes used in the hydrolysis process has a significant effect on the resulted product (Chen et al., 2018). The effect of the dose of the enzyme used in the hydrolysis can be seen in Fig. 3b. The addition of the enzyme dose increased the sugar concentration to its optimum point, then tended to decrease. The inhibition of the hydrolysis reaction occurs when the solid content is high, and the enzyme dose is low. This is associated with the problem of mass transfer and reduction of available water. Reaction inhibitors affect reaction kinetics, leading to longer hydrolysis time, and reducing glucose productivity, which has a significant effect on the resulting product (Alimny et al., 2019). However, this inhibition can be avoided using higher doses of the enzyme. The need for higher enzyme doses could be due to the nonspecific adsorption of protein by lignin, as has been reported in previous studies (dos Santos et al., 2019). The cellulase enzyme dose of 0.25 FPU/g produced the highest reducing sugar, which was 8.19 g/L. This dose is then used in enzymatic hydrolysis with several treatment processes and the addition of surfactants. Considering the economic feasibility on an industrial scale, low doses are more often chosen compared to processes that require larger amounts of enzymes.

3.3. Effect of surfactant addition and sequential treatment processes

Fig. 4 exhibit the concentration of reducing sugar obtained by adding 2.5 g/L and 5 g/L surfactants, respectively using various treatment

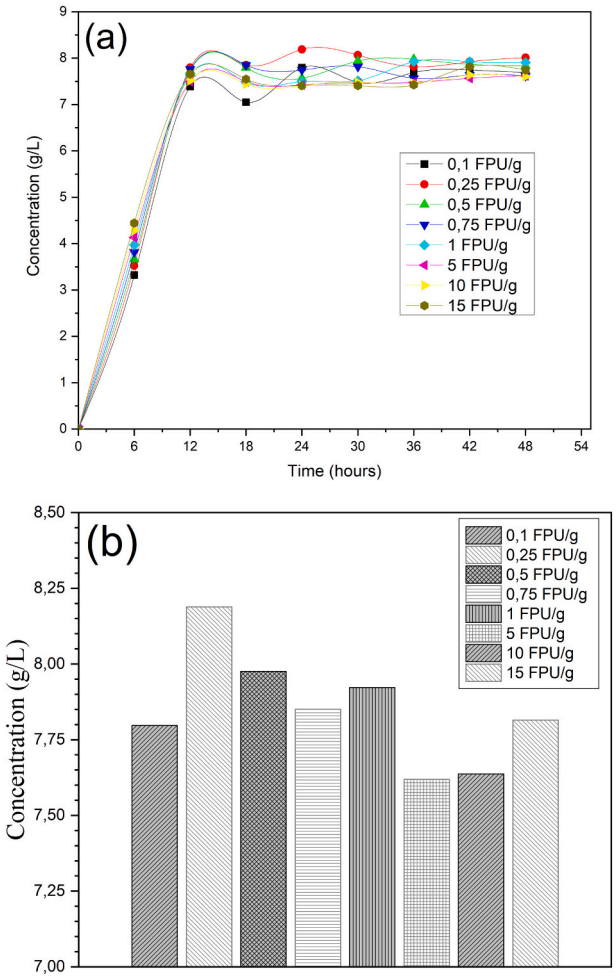

Fig. 3. Effect of time (a) and enzyme activity (b) on sugar production in the enzymatic hydrolysis process.

strategies. All Fig. 4 show the same trend i.e. the highest reducing sugar was obtained when the sample was treated with depectination and delignification, followed by without treatment, and finally with depectination treatment only. It can be seen in Fig. 4a and b that the untreated samples added with SDS surfactant produced higher yields than Tween-80 and PEG. This may be attributed to a high Hydrophilic–Lipophilic Balance (HLB) value of SDS. According to Lin et al. (2017), SDS can work optimally in increasing the sugar yield when lignin content is high by the addition of a low surfactant dosage. The positive effect of adding SDS was also reported by Muharja et al. (2019) on the hydrolysis of coconut coir which has high lignin content. The highest reducing sugar concentration was added when 2.5 g/L and 5 g/L surfactant were added at 5.80 g/L and 6.89 g/L, respectively.

The decrease in cellulase activity during enzymatic hydrolysis was caused by irreversible adsorption and inactivation of cellulase to cellulose. The addition of surfactants during enzymatic hydrolysis is an efficient method to minimize surface tension. Surfactants are liquid-soluble active surface agents that can reduce surface tension and increase the efficiency of cellulase adsorption to cellulose. PEG is a type of surfactant that can increase the efficiency of enzymatic hydrolysis when the lignin content is still present in the substrate. PEG works effectively at 50 °C. The increase in hydrolysis efficiency is due to the adsorption of PEG on lignocellulosic so that hydrophobic interactions occur, and hydrogen bonds are formed between PEG and lignin. The resulting hydrated layer will act as a steric barrier that can prevent cellulase from binding to lignin. Therefore, more cellulose will be hydrolyzed (Muharja et al., 2019). On the other hand, Tween-80, which is a nonionic

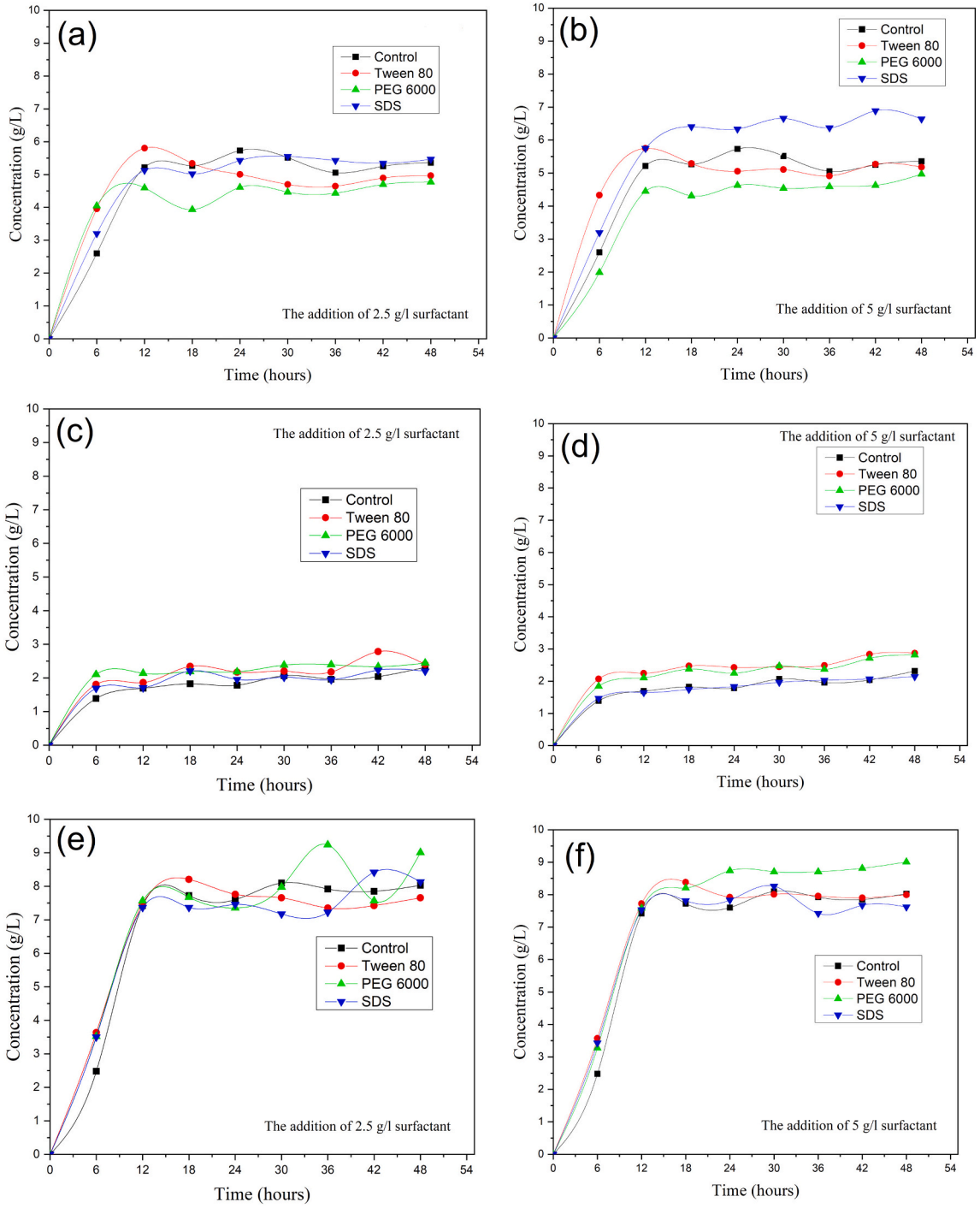

Fig. 4. The effect of various treatments on sugar recovery in enzymatic hydrolysis without treatment ((a) and (b)), with depectination ((c) and (d)), and with depectination and delignification ((e) and (f)) by the addition of 2.5 and 5 g/L surfactants, respectively.

surfactant as well, can decrease the adsorption of cellulase on the substrate because Tween-80 adsorbs a partially hydrophobic surface on lignin (Liu et al., 2020). Nonionic surfactants have a high affinity which can reduce the adsorption of cellulase with lignin so that the activity of cellulase enzymes can increase. Nonionic surfactants can effectively increase the efficiency of lignocellulose enzymatic hydrolysis, while anionic and cationic surfactants can inhibit enzymatic hydrolysis if used in excess. Anionic surfactants such as SDS can not only increase the efficiency of enzymatic hydrolysis in the concentration range of 0.1–1 mM, but also become inhibitors at concentrations higher than 1 mM (Lin et al., 2017).

The effect of depectination treatment with the addition of 2.5 g/L and 5 g/L surfactants can reduce reducing sugars (see Fig. 4c and d). This is because during the pectin extraction pectin polymer was destructed so that some sugar was easier to be released from CPH particles, causing a decrease in reducing sugars obtained in the enzymatic hydrolysis (Ninga et al., 2018). Based on previous work, it was reported that the depectinated substrate (without delignification) still contained lignin compounds (Muharja et al., 2021). The lignin causes the hydrolysis process to take place not optimally because the enzymes are blocked from accessing the cellulose, consequently reducing the sugar recovery during the hydrolysis process (Mao et al., 2021). The

depectination treatment resulted in the highest reducing sugars with the addition of 2.5 g/L and 5 g/L Tween 80 surfactant, namely 2.78 g/L and 2.87 g/L, respectively.

The use of an alkaline solution in the form of NaOH in the delignification process has been shown to increase the levels of the cellulose component (Muharja et al., 2021). The effectiveness of microwave delignification can also be enhanced by the addition of other additives. Previous studies have reported that the addition of surfactants, the use of microwave-assisted hydrotropic, and microwave-assisted deep eutectic solvents (DES) improved lignin removal significantly (Ma et al., 2021; Nababan et al., 2022). The addition of hydro tropes reduces surface tension and increases lignin solubility (Kłosowski et al., 2022). Microwave radiation can optimize the ionic characteristics of DES connective tissue to the maximum, increase its molecular polarity, reduce pretreatment temperature and time during the pretreatment process (Isci et al., 2020; Ma et al., 2021).

The effects of the addition of 2.5 g/L and 5 g/L surfactants on the depectinated and pretreated samples are shown in Fig. 4e and f. The addition of surfactant produced the highest reducing sugar when using PEG 6000. The sugar concentration is 9.24 g/L and 9.01 with the addition of 2.5 g/L and 5 g/L of surfactant, respectively. This is because PEG 6000 has a higher HLB value than Tween 80. A high HLB is good for extracting hydrophobic degradation products from lignin and hemicellulose. Nonionic surfactants such as PEG 6000 and Tween-80 cause an increase in the active site of cellulose so that the enzyme becomes easier to interact with a wider surface and increases the efficiency of enzymatic hydrolysis (Muharja et al., 2019). The addition of SDS surfactant to the enzymatic hydrolysis with delignification is less effective. This is because there has been a decrease in the amount of lignin on the substrate and the HLB value is too high (HLB SDS 40.0) (Ninga et al., 2018). Surfactants gave a significant effect on the hydrolysis process, especially in samples without delignification. This is because under these conditions, surfactants may increase the adsorption of cellulase on the substrate due to the adsorption of surfactants to lignin. This phenomenon does not relate during hydrolysis with depectination and delignification because lignin has been removed after delignification (Muharja et al., 2020b, 2020a, 2017). Thus, the concentration of reducing sugars from the addition of 2.5 g/L and 5 g/L surfactants did not differ much because the surfactants occupying the hydrophobic surface of the lignin were reduced during the delignification process. Without the addition of surfactants, inhibitory compounds such as phenols and tannins can affect the hydrolysis process. Phenol is a compound produced from the degradation of lignin. Phenol is the main inhibitor that can cause enzymes to become inactive. The absorption of tannins by cellulase enzymes can be prevented by adding surfactants during the enzymatic hydrolysis (Sjulander and Kikas, 2020). Although the addition of surfactant can bind the inhibitor, there is still the possibility that some enzymes become inactive due to absorbing the inhibitor. The nonactive cellulase enzyme causes the sugar concentration resulting from the hydrolysis process to be small (Muharja et al., 2019).

The addition of surfactant to the untreated and depectinated CPH samples had no significant effect on reducing sugar enzymatic hydrolysis (P-value > 0.05). On the other hand, the addition of surfactant with both depectination and delignification treatment had a significant effect on reducing sugar enzymatic hydrolysis (P-value < 0.05). Overall, variations in the treatment of CPH had a significant effect on the concentration of reducing sugars obtained from enzymatic hydrolysis. In the enzymatic hydrolysis process, adding 5 g/L of SDS surfactant to the untreated sample could increase sugar production by 20.1 % and produce the highest sugar concentration of 6.89 g/L. Chang et al. (2017) reported that the addition of 4 mM SDS could increase the efficiency of enzymatic hydrolysis of corn stover. This is because components such as lignin and corn stover ash non-productively adsorb some of the surfactants. In addition, the surface charge of SDS surfactant is positive, which can control the negative charge of the substrate and significantly improve the efficiency of enzymatic hydrolysis.

Nonionic surfactants such as Tween 80 and PEG 6000 are more effective in enzymatic hydrolysis of lignocellulosic biomass than anionic surfactants. In this case, the addition of Tween 80 or PEG 6000 will bind the surfactant to the lignin surface, leaving more free cellulase capable of hydrolyzing the cellulose chain. The increase in cellulose hydrolysis occurred along with the increase in surfactant dose. On the other hand, when the surfactant has exceeded the active site of lignin, the amount can interact with enzymes that can block its hydrolytic activity, resulting in a decrease in glucose yield caused by the addition of surfactant (Muharja et al., 2019). The highest sugar concentration in the samples treated with depectination was found in the addition of 5 g/L surfactant Tween 80, which was 2.87 g/L (an increase of 24.04 %). Previous studies also reported that the addition of surfactant Tween 80 in the enzymatic hydrolysis of coconut husks could increase the conversion of cellulose into reducing sugars by 30 % (Nogueira et al., 2018). Meanwhile, for samples that were treated with depectination and delignification, the highest sugar yield was 9.24 g/L when 2.5 g/L PEG 6000 was added, and the production increased by 14.07 %.

3.4. Cell growth and reducing sugar consumption

Fig. 5 showed the cells grew slowly during 0–12 h and the sugar was not significantly consumed. Cocoa pod husk hydrolysate contained the fermentable reducing sugars, but mixed with inhibitors which required adaptation for bacteria to grow. In this experiment, the strain grew in the short lag phase compared with the previous study which take up to 24 h lag phase. This was contributed by the addition of L-cysteine as the reducing agent (Liu et al., 2017). L-cysteine accelerated acids reassimilation in the lag phase (Chandgude et al., 2021). Similar condition in *C. perfringens* growth by phytic acid addition, cell growth was favoured and lag phase was shortened (Bloot et al., 2022). Cocoa pod husk hydrolysate with PEG 6000 also reduced the mass transfer resistance so that the hydrolysate adsorption into the cells and beads was fastened.

From 12 to 24 h, the cell growth increased significantly, up to 2.4 g/L DCW using immobilized cells with L-cysteine. The strain had adapted to the environment and the hydrolysate as the main substrate so they grew rapidly entering the log phase (Liu et al., 2017). The use of L-cysteine favoured the cell growth especially in the log phase as it helped the hydrogen chain circulation of NADH to NAD^- so that the formation of acetyl co-A and the pyruvate derivatives was higher (Chandgude et al., 2021).

Starting from 24 h, cell density did not increase, the strain began the stationary phase where some cells were dying due to the secondary metabolite production. It was caused by the higher acid concentration

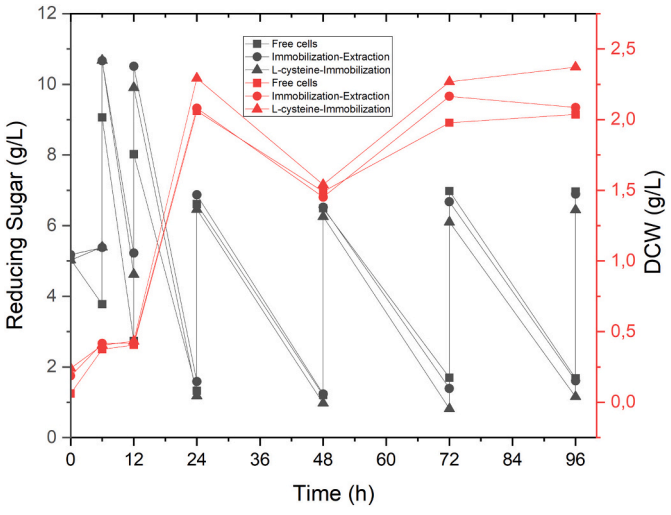

Fig. 5. Cell density and reducing sugar concentration in the extractive fermentation of cocoa pod husk hydrolysate.

and the formation of acetone, butanol, and ethanol during mid-high exponential phase. ABE production from mid-high exponential phase influenced pH of the media to base condition, shifting from acidogenic to solventogenic phase. pH of the samples as shown in Table 1 increased and the pH was buffered using calcium carbonate about 5 or above to lengthen the stationary phase for ABE production (Darmayanti et al., 2021).

3.5. Fermentation products

Fig. 6 shows the ABE concentrations using immobilized cells were higher than ABE concentrations in the fermentation using free cells. Using immobilized cells and the one with L- cysteine, the ABE concentrations were larger than the acids in the aqueous phase. This condition was caused by the use of immobilization protecting the cells from the toxic environment and favouring cells growth. Cells growth with higher rate is necessary to shift acidogenesis to solventogenesis by metabolizing glucose more effectively as energy source. Free cells with lower cell density than immobilized cells, consumed less sugar so that acidogenesis to solventogenesis shifting was slower (Darmayanti et al., 2020). In the extractant, butanol, acetone, and ethanol products were higher than the acids. The extraction in fermentation using free cells resulted in low ABE in the extractant because the ABE concentration in the media was also lower. On the other hand, ABE concentration of the extractant in the fermentation using immobilized cells were higher than the acids, with the highest butanol concentration of 17 g/L. Palm oil extractant was selective to the butanol than the other products.

Table 2 shows K_d and E in the fermentation using immobilized cells was higher than free cells, while the highest K_d and E of butanol was obtained using immobilized cells with L-cysteine. The K_d varied from 4.4 to 6.2 and E varied from 81.52 to 86.03 %. The higher value of K_d of butanol indicates the lower toxicity level to the microorganism (Khedkar et al., 2020). When the butanol concentration was high, the oxygen in the media was higher and toxic for anaerobic strain. Extraction in fermentation using immobilization gave higher K_d than free cells, while extraction using L-cysteine gave higher K_d than without L-cysteine. This phenomenon was affected by the pH of the media, so the butanol transferred into the organic phase was larger (Bidondo et al., 2021).

Table 3 shows total fermentation products in the aqueous and extractant phase. The ratio of total products (butanol:acetone:ethanol: acetic acid:butyric acid) using fermentation with free cells was about 1.2:1:1:1:1. Higher solvent products ratio was obtained using fermentation with immobilized cells with L-cysteine, with the value of 2:1.8:1.5:1:1. The addition of L-cysteine maintained acidity of the media so the solventogenic phase was optimized (Chandgude et al., 2021). In addition, L-cysteine protected from high acid concentration which disturbed the cells healing and maintained cells which require ATP to strengthen the active membrane permeability (ATPase). Consequently, high ATP was formed and the ATP requirement for cells healing during acidogenesis to solventogenesis shift was reduced (Chandgude et al., 2021).

Further about L-cysteine, an amino acid which was dissolved in the aqueous phase and not in the organic phase, so this reducing agent could help the activity effectively for the cells in the media and also the immobilized cells in beads. It reduced the redox potential of fermentation media, this is a characteristic of anaerobic fermentation that increases intracellular NADH. The reducing agent converted NAD^+ to NADH by electron donation. It happened if the redox potential was under −316 mV, NADH would be regenerated. L-cysteine with redox potential of −340 mV enhanced NADH regeneration causing the improvement of butanol production. The increase of NADH level contributed to the increase of enzyme activity, such as butanol dehydrogenase (BDH) butyraldehyde dehydrogenase (BADH), the enzymes responsible for the formation of butanol which were dependent to NADH (Chandgude et al., 2021). This resulted in a higher butanol production than the other solvents. As shown in Table 4, previous studies used free and immobilized cells, immobilized cells were used to protect the strain from the toxic environment and the fermentation products. Batch fermentation using immobilized cells reached butanol concentration up to 21.9 g/L compared with other studies with batch fermentation (Gedam et al., 2019). Immobilization demonstrated several advantages such as easing the cells separation from the products, higher cells density, and require smaller volume of fermentor for a higher productivity. Therefore, immobilized cells were used in this study to improve the cells' viability and productivity of the solvents.

Table 4 shows the result of butanol production in this study and previous studies. Butanol concentration in this study was higher than reports used free cells and the feed other than pure glucose (Jin et al., 2019; Sandesh et al., 2020). This difference might be because of cells immobilization favouring the strain so the butanol production was optimized. This was also proved by the other studies using immobilized cells (Darmayanti et al., 2018; Gedam et al., 2019; Menchavez and Ha, 2019). The table also shows that L-cysteine enhanced the performance during fermentation. The supplementation could improve the dehydrogenase activity (Lu et al., 2017). The additives reported using Tween 80 and L62 as surfactant dissolved hydrophobic compound such as butanol which is toxic for the strain (Gedam et al., 2019). The reduction of toxicity of the products was proved to enhance butanol production. The extractive fermentation was utilized to harvest the products simultaneously so that the products' toxicity in the media was reduced, resulting in a longer period of solventogenesis (Gedam et al., 2019).

Table 4 also shows the effect of feed type to ABE yield. Abe production from CPH hydrolysate in this study was higher than other studies using hydrolysate of sugarcane bagasse, corn pulp, and apple pulp (Jin et al., 2019; Wang et al., 2020). Although the ABE concentration in this study were lower than fermentation using pure glucose (Menchavez and Ha, 2019), this system could be a competitive process. This condition was due to hydrolysate of lignocellulosic feedstock had different sugar composition. Other sugars and polysaccharide such as cellulose, hemicellulose, and xylose required certain enzyme and different intracellular NADH concentration. The strain needed adaptation to produce those enzymes and requires genetic modification to increase the activity of the enzymes. However, this study proved that the addition of reducing agent (L-cysteine) and immobilization helped the fermentation using cocoa pod husk as the feedstock. This provided simpler alternative and consumes lower cost than genetic modification for the strain to produce ABE from CPH where the hydrolysate was considered as economical feedstock with the cost about half of the pure glucose (Wang et al., 2020).

Table 1
pH of the fermentation media.

Time (h)	Free cells	Immobilized cells	Immobilized cells with L-cysteine
0	5.18	5.03	5.10
6	4.93	5.75	5.82
12	5.38	5.17	5.04
24	5.39	5.02	4.95
48	6.54	5.12	5.32
72	6.38	4.77	5.60
96	6.82	4.90	5.40

4. Conclusions

The sequential treatment and the addition of surfactants have been proved to increase the production of reducing sugars. The highest concentration of reducing sugar in enzymatic hydrolysis was obtained by sequential depectination and delignification, followed by without pectin extraction and delignification with the addition PEG 6000, and finally by pectin extraction only. ABE production by extractive fermentation was enhanced using immobilized cells and L-cysteine supplementation. Immobilization protected the strain so that the cells adapted faster to the environment and more viable during ABE production. L-cysteine

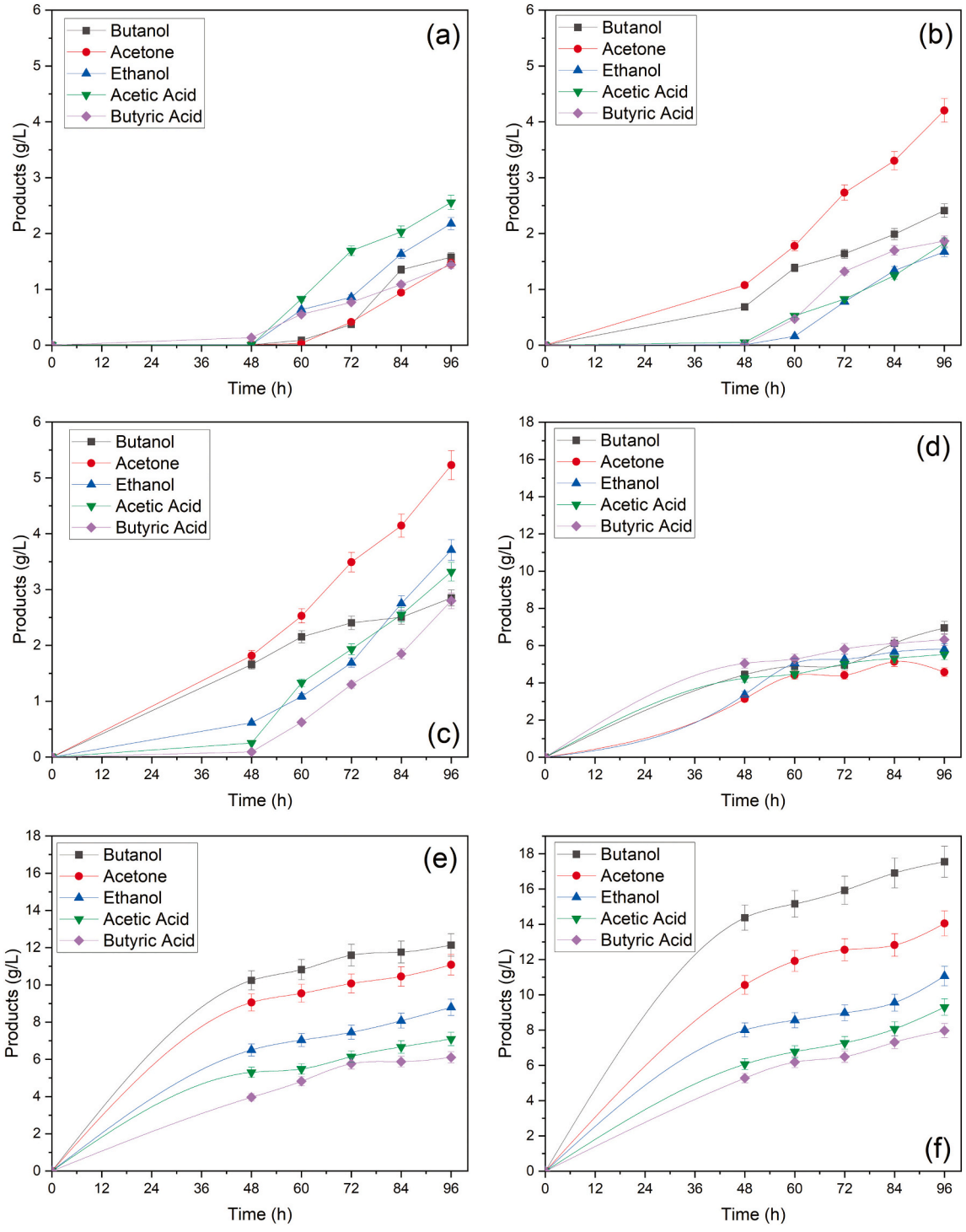

Fig. 6. Products time course in the media (a–c) and extractant (d–e) of extractive fermentation using free cells (a and d), immobilized cells (b and e), immobilized cells with L-cysteine (c and f).

Table 2
Distribution coefficient (K_d) and extraction efficiency (E).

Cells type	Distribution coefficient (K_d)					Butanol extraction efficiency (%)
	BuOH	Acetone	Ethanol	Acetate	Butyrate	
Free	4.410	3.110	2.667	0.317	4.381	81.52
Immobilized	5.036	2.635	5.264	0.205	0.234	83.43
Immobilized – with L-cysteine	6.159	2.688	2.983	2.804	0.260	86.03

Table 3
Total products concentration in aqueous and extractant phase for several cells type.

Time (hour)	Butanol (g/L)			Acetone (g/L)			Ethanol (g/L)			Acetic acid (g/L)			Butyric acid (g/L)		
	F[a]	I[b]	IL[c]	F[a]	I[b]	IL[c]	F[a]	I[b]	IL[c]	F[a]	I[b]	IL[c]	F[a]	I[b]	IL[c]
48	4.436	10.927	16.030	3.146	10.129	12.377	3.376	6.510	8.623	4.241	5.356	6.323	5.183	3.977	5.372
60	4.972	12.205	17.314	4.441	11.324	14.452	5.683	7.194	9.646	5.303	6.011	8.106	5.834	5.293	6.812
72	5.339	13.233	18.330	4.826	12.808	16.048	6.130	8.231	10.671	6.702	6.973	9.207	6.583	7.081	7.794
84	7.473	13.749	19.413	6.074	13.749	16.968	7.292	9.414	12.312	7.349	7.911	10.619	7.206	7.572	9.165
96	8.523	14.550	20.395	6.037	15.280	19.277	7.982	10.462	14.770	8.077	8.920	12.626	7.760	7.970	10.765

[a] Fermentation by free cells.
[b] Fermentation by immobilized cells.
[c] Fermentation by immobilized cells with L-cysteine addition.

Table 4
Comparison with previous studies.

No.	Species[a]	Fermentation mode	Cells	Additive	Feedstock	Total concentration (g/L)		Reference
						BuOH	ABE	
(1)	Ca	*Batch*	Free	–	Sugarcane bagasse	7.4	13	(Pratto et al., 2020)
(2)	Ca	*Batch*	Free	–	Corn pulp	6.9	10.3	(Wang et al., 2020)
(3)	Cb	*Batch*	Free	–	Apple pulp	6.3	10.7	(Jin et al., 2019)
(4)	Ca	*Fed-batch*	Immobilized	–	Glucose	21.6	26.8	(Menchavez and Ha, 2019)
(5)	Cs	*Fed-batch*	Immobilized	–	Glucose	64.6	97.3	(Darmayanti et al., 2018)
(6)	Ca	*Batch*	Free	–	Cocoa pod husk	11.73	18.25	(Sandesh et al., 2020)
(7)	Csp	*Batch*	Immobilized	*Surfactant* L62	Glucose	21.9	21.9	(Gedam et al., 2019)
(8)	Cb	*Batch*	Free	L-cysteine	Fructose	11.3	11.3	(Lu et al., 2017)
(9)	Cs	*Fed-batch*	Immobilized	L-cysteine	Cocoa pod husk	20.4	54.4	This study

[a] Ca: *Clostridium acetobutylicum*, Cs: *Clostridium saccharoperbutylacetonicum* N 1–4, Cb: *Clostridium beijerinckii*, Csp: *Clostridium sporogenes*, Cb: *Clostridium Beijerinckii*.

increased the metabolism of the substrate and butanol production by improved enzyme activity. This study is an interesting alternative for cocoa pod husk utilization to produce ABE with simple technology, reusable immobilized cells, and economical materials.

CRediT authorship contribution statement

All authors contributed to the study's conception and design. Material preparation, data collection and analysis were performed by Istiqomah Rahmawati, Helda Wika Amini, Ditta Kharisma Yolanda Putri, and Meta Fitri Rizkiana. The validation, visualization, and writing the original draft was executed by Maktum Muharja and Rizki Fitria Darmayanti. Bekti Palupi, Felix Arie Setiawan, Arief Widjaja, and Abdul Halim carried out review and editing. Boy Arief Fachri managed and supervised this work. All authors read and commented on previous versions of the manuscript and approved the final manuscript.

Declaration of competing interest

All authors declare that they have no conflicts of interest.

Data availability

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

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