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Effect of medium supplementations and extraction conditions on cellulase production through solid state fermentation of oil palm empty fruit bunches

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ABSTRACT In this study, cellulase enzyme was produced through solid state fermentation (SSF), employing oil palm empty fruit bunches (EFB) as the primary substrate. Two key aspects were explored to enhance crude enzyme yield, which are medium supplementation effects during SSF and cellulase recovery. During medium supplementation, glucose and/or Tween 80 were added alongside EFB substrate and other nutrients. Enzyme yield was determined using a filter paper assay and expressed as enzyme activity. The initial addition of glucose during fermentation led to increased crude enzyme activity, as measured by the filter paper assay. The peak crude enzyme activity was observed with the addition of 3 mg of glucose, with higher amounts showing no further increase in activity. Conversely, the addition of Tween 80 did not yield any significant increase in crude enzyme activity across all concentrations tested. The extraction conditions were varied to study cellulase recovery, specifically by adjusting the solid-to-solvent ratio and the number of extraction stages. Higher enzyme activity was achieved with lower solid-to-liquid ratios, as the increased solvent volume facilitated greater enzyme extraction. However, increasing the number of extraction steps did not significantly affect the resulting cellulase activity. Overall, this research underscores the need for further process optimization for cellulase production via SSF, utilizing the widely available EFB in Indonesia.

KEYWORDS Cellulase; Oil palm empty fruit bunches; Solid state fermentation

1. Introduction

The production and utilization of bioethanol as an alternative to renewable energy continue to increase every year. Bioethanol can be generated from renewable sources such as sugar, starch, lignocellulosic biomass, and algae. Of all the alternatives, lignocellulosic biomass tends to be the most promising, as it can be used as bioethanol feed without interfering food and feed production (Zabed et al. 2017). To produce bioethanol, lignocellulose has to be hydrolized into glucose monomers. One of the enzymes that is required for such process is cellulase (Kuhad et al. 2016). However, high cost of cellulase production frequently becomes an obstacle to synthesize bioethanol from agro-industrial waste biomass as a substrate. This cost is estimated to reach around 40% of the total cost of bioethanol production (Behera and Ray 2016). Therefore, further innovation and studies are needed to reduce the cost of cellulase enzyme production.

Cellulase is a complex enzyme that consists of three enzymes, which are endoglucanase (E.C.3.2.1.4), exoglucanase (E.C.3.2.1.9), and β-glucosidase (E.C.3.2.1.21). The deconstruction of cellulose is initiated by endoglucanase, which randomly attacks amorphous sites in the cellulose chain, resulting in long-chain oligosaccharides. Exoglucanase, on the other hand, targets the crystalline regions in the cellulose chain to produce short-chain oligosaccharides. Finally, β-glucosidases hydrolyze these oligomers into glucose (Verma et al. 2021). Current commercial cellulase production focused on the use of submerged fermentation (SmF) method using fungi as the producer microorganisms, usually from the genus *Trichoderma* or *Aspergillus* (Singh et al. 2021). This method used cellulose substrate dispersed in liquid phase, so the enzyme purification process is costly (Yoon et al. 2014). Alternatively, solid state fermentation (SSF) method had gained attention (Behera and Ray 2016). Comparison of cellulase activity from *Trichoderma reesei* RUT-30 produced under SmF and SSF condition showed that the enzyme complex produced under SSF condition exhibited excellent activity compared to the one produced under

SmF condition (Prévot et al. 2013). Solid state fermentation allowed the utilization of agricultural waste with only minimal liquid addition, which mimics fungi natural environment. It allowed for lower substrate cost and downstream processing cost, which could potentially suppress cellulase price (Wonoputri et al. 2018; Singh et al. 2021). Numerous agricultural wastes have been studied as SSF substrate, such as rice straw and hulls, wheat bran, rice bran and husk, banana waste, sugarcane bagasse, apple pomace, corn stover, etc (Kang et al. 2004; Jo et al. 2008; Singh et al. 2021; Heng and Hamzah 2022). One agricultural waste that can be used to produce cellulase enzymes is empty oil palm fruit bunches (EFB), which are produced in the scale of millions of tons in Indonesia every year.

Previous studies have shown the potential of EFB as SSF feed in cellulase production (Wonoputri et al. 2018). To further increase the obtained cellulase activity, it is interesting to add different medium supplementation during fermentation. Medium composition and supplementation are known to be an influencing factor for promoting fungi growth and subsequent cellulase yield. Medium containing cellulose, yeast extract, and glucose, together with supplementation of Tween 80 resulted in higher dry *T. reesei* biomass weight, bigger total mycelia surface area, and better volumetric enzyme productivity compared to medium fermentation containing cellulose, yeast extract, peptone, and urea only (Ahamed and Vermette 2009). The addition of glucose as a secondary carbon source could promote mycelial growth needed for fermentation (Li et al. 2016) although it did not necessarily increase the final cellulase activity if used as the sole carbon source (Ju and Afolabi 1999; Niranjane et al. 2007). However, the amount of glucose should be carefully selected, as monosaccharide could limit cellulolytic enzyme expression (Jönsson and Martín 2016). Fortunately, catabolite repression by glucose in fungi was minimized when SSF was used compared to SmF, allowing high growth of fungus in the presence of high sugar concentration (Hölker et al. 2004). This shows the need for further study in utilizing glucose for SSF medium supplementation.

Medium supplementation using surfactants have been proposed as one of the strategies to improve cellulase yield (Passos et al. 2018). Surfactant could increase cellulase yield by different mechanisms, such as increasing microbial cell membrane permeability, promoting cell-bound enzyme release (Pardo 1996), improving cellulase stability, and preventing enzyme denaturation (Liu et al. 2006). Numerous surfactants have been reported to be successful in increasing cellulase production. For instance, the biosurfactant rhamnolipid were found to increase both cellulase and xylanase production by *Trichoderma viride* and *T. reesei* Rut C30 (Liu et al. 2006; Callow and Ju 2012). Chemical surfactant such as Tween 80 was also found to increase production of cellulolytic enzymes in *T. viride* and *Nectria catalinensis* (Pardo 1996; Liu et al. 2006). The stimulatory effect of Tween 80 was also reported on other fungal species, such as *Aspergillus terreus* (Shahriarinour et al. 2011). However, when Tween 20 and Triton X-100 were used, inhibition of cellulolytic enzyme production and fungal growth was observed in *N. catalinensis* (Pardo 1996). This clearly shows the potential of the widely available Tween 80 as cellulase production stimulant.

The obtained enzyme activity may also be affected by the extraction condition that is needed to recover the cellulase enzyme produced in SSF. Manipulation of extraction condition was even suggested as a simple strategy to enhance enzyme recovery from SSF. For instance, enhancement of pectinase recovery was achieved by optimizing extraction temperature, contact time, and solvent time (Castilho et al. 2000). Xylanase and exo-PG recovery was enhanced by 7.7 and 5.5 fold by optimizing type and volume of solvent, extraction temperature, incubation time, and stirring rate, and doing repetitive extraction (Díaz et al. 2007). Similarly, cellulase recovery from SSF was also enhanced when the solvent type, extraction mode, solid to solvent ratio, and number of extraction stages in co-current scheme were optimized (Marín et al. 2019). This indicates that enhancement of enzyme activity should not only focus on optimization of SSF parameter, but also be expanded to the recovery process.

This study focused on studying medium supplementation effect during SSF and recovery strategy for cellulase production. Glucose and Tween 80 were used as supplementation for SSF using untreated EFB as cellulose source. The produced enzyme was recovered by extraction using citrate buffer, where the effect of solid to solvent ratio and number of extraction stages were studied. Cellulase production was quantified by filter paper assay and expressed as enzyme activity.

2. Materials and Methods

2.1. Enzyme production

2.1.1 Solid medium preparation

This study used EFB obtained from PT Perkebunan Nasional VIII in Bogor, Indonesia. The EFB was washed to remove impurities and dried in an oven at 80 °C for 24 h, then grinded into the size of <0.5 cm. Three grams of EFB were put into a 250 mL Erlenmeyer and autoclaved (121 °C, 15 min) before being used as the main substrate in SSF process.

2.1.2 Inoculum culture

Trichoderma viride QM9414 fungi was used as a cellulase-producing microbe in this experiment. *T. viride* QM9414 were kept in the culture collection of Laboratory of Microbiology and Bioprocess Technology, Department of Chemical Engineering, Institut Teknologi Bandung. The ability of the fungi in producing cellulase has been previously reported (Ülger and Salam 2001) and importantly shown to have better productivity compared to the commonly used *T. reesei* NRLL6156 (Wonoputri et al. 2018). The fungi were grown on the surface of potato dextrose agar (PDA) at 30 °C and regenerated every seven days.

The spore suspension for the fermentation process was prepared by adding 6 mL of 0.9% NaCl into a slanted agar containing a 7-day-old *T. viride*. NaCl solution was selected to maintain the spore osmotic pressure. Then, 1 mL of the spore suspension was added to EFB to carry out the fermentation process. After that, each Erlenmeyer was supplemented with 3 mL of Mandels nutrient with the following composition (in 1 liter of aqua dm): 0.3 g urea, 0.1 g peptone, 1.4 g $(NH_4)_2SO_4$, 2 g KH_2PO_4, 0.3 g $CaCl_2.2H_2O$, 0.3 g $MgSO_4.7H_2O$, 5 mg $FeSO_4.7H_2O$, 1.6 mg $MnSO_4.H_2O$, 1.4 mg $ZnCl_2.7H_2O$, and 2 mg $CoCl_2.6H_2O$ (Mandels et al. 1962; Li et al. 2016).

2.1.3 Solid state fermentation

The study on effect of glucose concentration was carried out by adding 3, 6, and 9 mg of glucose (10 g/L; equal to 0.3, 0.6, and 0.9 mL of glucose) into the fermentation substrate. The glucose concentration that gave the highest cellulase enzyme activity was then used to test the effect of Tween 80 addition to the fermentation medium at 1%, 2 and 3% v/v (equal to 0.01; 0.02; and 0.03 g/g dry substrate). Fermentation was carried out for 5, 7, and 10 days for each variation. The variations that gave the highest cellulase activity were then used to test the effect of the extraction conditions. All experiments were conducted in duplicates and error bar denoted standard error between replicates. The results were analyzed statistically using one-way ANOVA with Dunnett post hoc analysis, where p-value < 0.05 was considered statistically significant.

2.2. *Extraction and analysis*

Cellulase enzyme was extracted from the fermentation medium using 50 mM citrate buffer, pH 4.8, as the solvent. At the end of fermentation, citrate buffer was added to the fermentation flask and mixed at 30 °C and 150 rpm for 2 h. The extract was filtered using filter paper under vacuum. Cellulase activity in the extract was analyzed by the filter paper assay (FPA) method, following the method described previously (Wonoputri et al. 2018). In brief, 0.4 mL of the crude cellulase enzyme solution was added to a test tube containing 1 mL of citrate buffer and Whatman No. 1 filter paper with size of 1 × 6 cm. The test tubes were then incubated at 50 °C for 60 min before adding 3 mL of DNS reagent to react with the reducing sugars produced during incubation. Enzyme activity was expressed as FPU/mL stating the amount of enzyme required to produce 2 mg of reducing sugar during the hydrolysis process. The FPU/mL unit was then converted to FPU/g substrate, where 1 FPU/mL was equivalent to 8 FPU/g substrate.

The effect of solid to solvent ratio and number of extraction steps were studied using counter-current scheme, as illustrated by Figure 1. The solid to liquid ratio were varied between the standard condition of 1:8 (total solvent used 24 mL) or 1:10 (total solvent used 30 mL). Number of extraction steps were varied between 1, 2, 3, and 4 stages of extraction following the counter-current scheme, where the filtrate from the previous extraction stage was used as an extracting solution for the next extraction stage. All experiments were conducted in duplicates.

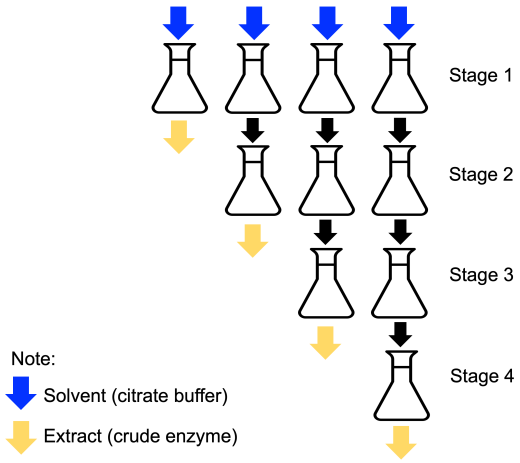

FIGURE 1 Experimental scheme for variation in solid to solvent ratio and number of extraction steps. The blue arrows denote fresh solvent, while the yellow arrows denote the extract.

3. Results and Discussion

3.1. *Effect of glucose on crude cellulase activity*

The activity of crude enzyme from the control system increased significantly on day 5 and continued to increase to reach its maximum value on day 10. On the other hand, when 3, 6, or 9 mg of glucose was added as the secondary carbon source, the highest enzyme activity was achieved faster on day 7 (Figure 2). Glucose supplementation had been shown to increase microbes' growth rate (Bischof et al. 2013), which correlated to higher fungal mycelial growth needed for enzyme production (Li et al. 2016). Consequently, the addition of glucose also increased the maximum crude enzyme activity. In here, supplementation with 3 mg of glucose increased crude enzyme activity to reach a maximum value of 0.373 FPU/g, which was

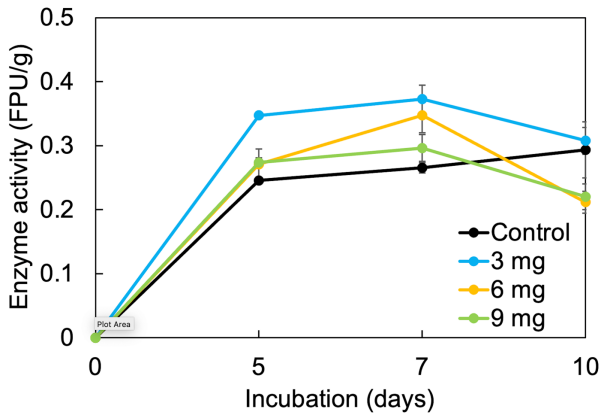

FIGURE 2 Effect of glucose addition on cellulase productivity (expressed as enzyme activity) for control (without the addition of glucose; black line) and with the addition of 3 mg (blue line), 6 mg (yellow line), and 9 mg (green line) of glucose.

significantly higher (p-value < 0.05) than the maximum enzyme activity obtained for control (0.294 FPU/g). Similarly, supplementation with 6 mg of glucose also increased the maximum crude enzyme activity (0.347 FPU/g; p-value = 0.1), although at a lower level compared to 3 mg of glucose supplementation. Increasing supplementation with 9 mg of glucose did not increase the activity further, whereby similar enzyme activity as the control (0.296 FPU/g) was observed. It suggested that the addition of glucose at a suitable concentration could increase cellulase production.

Although glucose supplementation has been shown to increase fungal growth and cellulase production (Li et al. 2016), the presence of too much glucose could result in the opposite effect due to catabolite repression. Catabolite repression is a regulatory mechanism frequently observed in bacteria and fungi, which inhibits the activation of genes needed for utilizing alternative carbon sources when a preferred substrate is available. This means that when cellulose and glucose are both present in the fermentation system, the fungi metabolize glucose and stop the utilization of cellulose, thereby halting the subsequent production of cellulase (Nair and Sarma 2021; Verma et al. 2021). Moreover, glucose and other simple sugars also could be present as cellulose hydrolysate product from cellulase activity (Adnan et al. 2018). This usually caused the decrease in cellulase activity at longer fermentation time. As observed here, after the maximum enzyme activity was reached at 7 days, and fermentation was continued to 10 days, the enzyme activity detected from systems with the addition of glucose decreased. Additionally, several reports have suggested that glucose inhibition on cellulase could also happen through different mechanism other than catabolite repression ((Xiao et al. 2004; Hsieh et al. 2014). Previous studies showed that high glucose concentrations inhibit cellulase adsorption on the hydrolysis substrate, consequently decreasing cellulase hydrolysis yields (Hsieh et al. 2014). This implied that determination of optimum fermentation duration was important to obtain the maximum cellulase activity.

3.2. *Effect of Tween 80 in cellulase activity*

The effect of Tween 80 on crude cellulase activity was also conducted. In the control condition (without the presence of Tween 80, but with the addition of 3 mg of glucose), the highest cellulase activity was obtained after seven days of incubation (Figure 3). The addition of 1% of Tween 80 seemed to suggest that there was a slight increase (∼6%) after seven days of fermentation, however this was not confirmed by statistical analysis. When 2% and 3% of Tween 80 was used in the fermentation, the maximum enzyme activity was achieved on day 5 instead, where the maximum enzyme activity was 0.359 and 0.324 FPU/g, respectively. Statistical analysis showed that only the variation with 3% of Tween 80 addition was statistically significant (p-value < 0.05). This indicates that not only Tween 80 addition did not significantly increase cellulase activity, but a significant reduction at high concentration of Tween 80 was observed.

Tween 80 at a range of 0.05 to 0.15% (w/w) could increase cellulase and xylanase production by *T. viride* in SSF on rice straw, bran, and sawdust combination as the substrate (Liu et al. 2006). Similarly, Tween 80 addition of 1% (v/v) had been reported successful in increasing cellulase production by *T. reesei* (Hari Krishna et al. 2000), which was within the range of Tween 80 concentration used in this study. In fact, most studies had noted the effectiveness of Tween 80 addition in increasing cellulase yield (Shahriarinour et al. 2011), which was not observed in this study. Tween 80 toxicity toward fungi had not been reported before, and Tween 80 addition was reported to give minimal effect towards fungi growth, even when the cellulase yield was already maxed. In this study, to keep the moisture level at the same level, Tween 80 addition consequently reduced the amount of liquid medium/nutrient added into the fermentation medium. Thus, the absence of Tween 80 positive effect in this study could only be correlated to the reduction in liquid nutrient.

The presence of Tween 80 could potentially increase cellulase yield if added independently at a low concentration such that it does not affect moisture level used in SSF. For instance, 0.15% of Tween 80 addition increased cellulase enzyme activity produced by *T. viride* up to 38% compared to fermentation without Tween 80 addition (control) (Liu et al. 2006). The increase in cellulase enzyme yield after Tween 80 addition was also obtained when *Penicillium simplicissimum* was used. Besides that, 0.05% of Tween 80 addition obtained an increase in cellulase enzyme activity 44% higher than the fermentation without Tween 80 addition (Zeng et al. 2006). This indicates that Tween 80 addition still holds the potential to increase enzyme yield, as long as the suitable condition is fulfilled. This would be the focus of our future study.

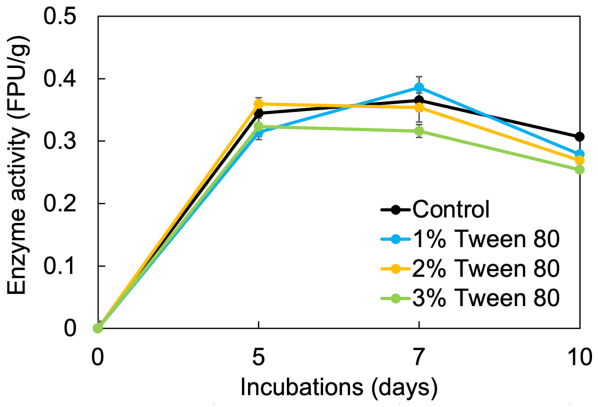

FIGURE 3 Effect of Tween 80 addition on cellulase productivity (expressed as enzyme activity) for control (without the addition of Tween 80; black line) and with the addition of 1% (blue line), 2% (yellow line), and 3% (green line) of Tween 80.

3.3. *Extraction condition variation on crude cellulase activity*

Variations of enzyme extraction condition were conducted using a counter current extraction scheme (Figure 1) simultaneously. The result for variations in solid to solvent ratio and number of extraction steps is presented (Figure 4). The optimum solid to solvent ratio have been found to be different depending on the solid substrate characteristics. For instance, cellulase from apple pomace and rice fiber fermentation can be extracted using the smallest solid to solvent ratio of 1:2 and 1:1, respectively. On the other hand, cellulase from orange peel fermentation must operate at solid to solvent ratio of 1:3 due to larger particle size compared to the other two substrates (Marín et al. 2019). Due to the large porosity and high-water retention capability of EFB, solid to solvent ratio of 1:8 (standard condition) and 1:10 were employed. Experimental results (Figure 4) shows that when higher solid to liquid ratio of 1:10 (corresponds to 30 mL of buffer used) for the extraction process, the final enzyme activity that can be obtained was around 0.434 FPU/g. This was significantly higher than using solid to liquid ratio of 1:8 (0.327 FPU/g; p-value < 0.05). The positive effect of the solid to liquid ratio on enzyme activity was influenced by the mass transfer process facilitated by the difference in the concentration gradient between the solid (substrate) and liquid (solvent) phases (Pirota et al. 2013). In addition, under conditions of higher buffer volume, more cellulase enzymes could be extracted because the total allowable soluble enzymes in the citrate buffer increased (although the saturation of the citrate buffer did not change).

Higher enzyme activity that can be obtained at lower solid to liquid ratio has been observed before. For instance, extraction of endoglucanase at 160 rpm and 35 °C was conducted, and the enzyme activity measured at a solid to liquid ratio of 1:3 and 1:9 were 21.6 IU/g and 35.7 IU/g, respectively (Pirota et al. 2013). Although the final enzyme activity obtained was slightly higher at a ratio of 1:10, the total volume of the solution needed should be considered, as when more solvent was used, it might not be beneficial economically.

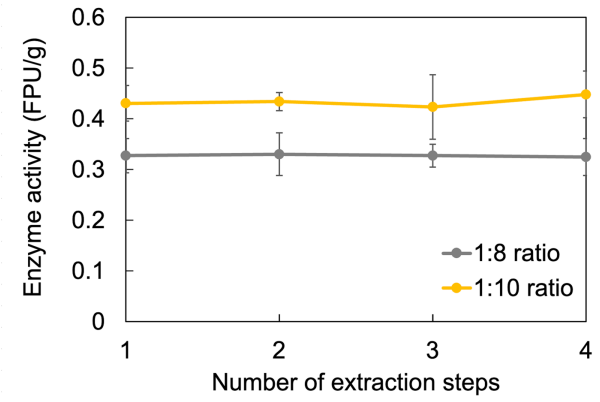

FIGURE 4 Effect of extraction condition variations on obtained crude cellulase recovery (expressed as enzyme activity). Solid to liquid ratio of 1:8 (grey line) and 1:10 (yellow line) were used.

The increase in extraction stages did not show a significant effect on the obtained crude activity, whether using one step of extraction or even after four steps of extractions. The final value of enzyme activity obtained at all variations of the extraction steps were quite similar, ranging from 0.325–0.330 FPU/g (for 1:8 ratio) to 0.423–0.448 FPU/g (1:10 ratio). This suggests that the use of sequential extraction was not necessary. In fact, most literature have reported that one extraction stage was already sufficient in recovering most of the cellulase produced in most cases (Pirota et al. 2013; Marín et al. 2019).

Several studies have investigated cellulase production through SSF using EFB as the substrate. For instance, previous research achieved a maximum cellulase activity of 0.76 U/mL using *Aspergillus terreus* (Shahriarinour et al. 2011). Additionally, the cellulase production by *Trichoderma harzianum* T2008 was examined both in Erlenmeyer flasks and a 50 L rotary drum bioreactor, resulting in maximum activities of 8.2 and 10.1 FPU/g dry solid, respectively. In contrast, the current study yielded a maximum activity of approximately 0.05 FPU/mL, which appears comparatively low, particularly when compared with commercial cellulase enzyme such as Cellic® Ctec 2 (119 FPU/mL) (Ju et al. 2014). However, it is noteworthy that previous research has demonstrated that crude cellulase enzymes produced via SSF using EFB as the substrate have better reducing sugar yields from filter paper compared to commercial enzyme blends (Zhu et al. 2014). Consequently, this research still holds substantial potential for further exploration and optimization.

4. Conclusions

This study assessed different strategy in increasing cellulase enzyme activity from SSF. The addition of minimal amount of glucose successfully increased the obtained crude cellulase activity by up to 40%. However, the presence of too much glucose or cellulose hydrolysate in the fermentation medium can result in a decrease in fermentation productivity. Moreover, the addition of surfactant Tween 80, which consequently reduced the amount of liquid medium in the SSF system, did not significantly increase cellulase enzyme activity. In fact, a significant decrease in cellulase activity (∼17%) was observed at the highest concentration of Tween 80 tested here.

Optimization in cellulase recovery process was also studied to improve cellulase yield. Among different factors, the solid to solvent ratio and number of extraction stages in counter-current extraction method were selected and studied. Lower solid to solvent ratio resulted in a positive result as higher crude enzyme could be obtained when solid to solvent ratio of 1:10 was used compared to the standard condition of 1:8. However, the selection of the solid to liquid ratio needs further optimization, as lower solid to liquid ratio will result in higher volume of crude enzyme that need to be processed. Conversely, increasing the number of extraction steps did not have any sig-

nificant effect on the resulting cellulase activity. Overall, this study showed the potential for advanced optimization study to increase cellulase yield from EFB using SSF.

Acknowledgments

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Authors' contributions

VW, RP designed the study. JW, JS carried out the laboratory work. JS, JW, VW, RP analyzed the data. VW wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no competing financial interest.

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