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Cellulase Addition and Pre-hydrolysis Effect of High Solid Fed-Batch Simultaneous Saccharification and Ethanol Fermentation from a Combined Pretreated Oil Palm Trunk

Afrasiab Khan Tareen, Vittaya Punsuvon, Imrana Niaz Sultan, Muhammad Waseem Khan, and Pramuk Parakulsuksatid*

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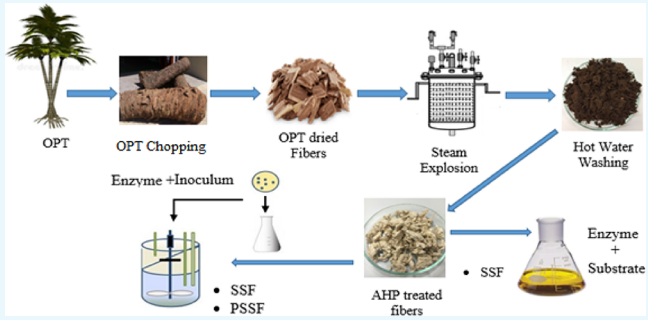
ABSTRACT: In the current study, alkaline hydrogen peroxide pretreated oil palm trunk fibers were subjected to ethanol production via simultaneous saccharification and fermentation (SSF). The effect of high substrate loading, enzyme and substrate feeding strategy, and influence of a pre-hydrolysis step in SSF was studied to scale up ethanol production. In the enzyme feeding strategy, the addition of an enzyme at the start of fed-batch SSF significantly ($p < 0.05$) increased ethanol concentration to 51.05 g/L, ethanol productivity (Q_P) to 0.61 g/L·h, and ethanol yield ($Y_{P/S}$) to 0.31 g/g, with a theoretical ethanol yield of 60.65%. Furthermore, the initial velocity of the enzyme (V_0) in the first 8 h was 2.27 (g/h) with a glucose concentration of 18.17 g/L. On the other hand, the substrate feeding strategy and pre-hydrolysis simultaneous saccharification and fermentation (PSSF) process were studied in a 1 L fermenter. PSSF in fed batch with 10 and 20% (w/v) significantly improved enzyme hydrolysis, circumvent the problems of high viscosity, reduced overall fermentation time, and gave the highest ethanol concentration of 51.66 g/L, ethanol productivity (Q_P) of 0.72 g/L·h, ethanol yield ($Y_{P/S}$) of 0.31 g/g, and theoretical ethanol yield of 60.66%. In addition, PSSF with 10 and 20% significantly increased the initial velocity of the enzyme (V_0) to 4.64 and 4.40 (g/h) and glucose concentration to 37.14 and 35.27 g/L, respectively. This result indicated that ethanol production by PSSF along with substrate feeding could enhance ethanol production efficiently.



1. INTRODUCTION

The intensive global energy consumption, sustainable energy, and depleting natural resources of fossil fuel urge the hunt for renewable energy sources. More specifically, fossil fuel utilization as the main energy source has caused several problems such as global warming and environmental pollution.[1] Lignocellulosic biomass is known to be one of the most plentiful renewable energy sources that also serve as an economical agriculture material for second-generation biofuel production. In renewable energies, liquid biofuels account for ∼40% of the entire world's energy consumption,[2] which contribute to reducing the emission of greenhouse gas.[3]

Oil palm trees (plentiful in Thailand) are industrially utilized for producing palm oil. For better productivity, replantation of oil palm trees at an interval of 25 to 30 years is necessary.[4,5] The replantation of oil palm trunk (OPT) creates heaps of biotrash at the sites of the plantation, which is mostly treated as agricultural waste by dumping or burning.[6]

Globally, oil palm biomass is produced and utilized in million metric tons annually, which generates more than 295 million tons of waste worldwide. In addition, Indonesia, which is a leading oil palm producer followed by Malaysia and Thailand, produces more than 100 million tons of palm biomass solid waste per year.[7] In 2019, Indonesia exported 29.52 million metric tons of palm oil while Malaysia exported 18.47 million metric tons.[8] Moreover, Thailand along with Nigeria and Colombia collectively produced 7% of oil palm in the world.[9] The oil palm trunk, a lignocellulosic material, is composed of interpenetrated polymeric components such as cellulose, hemicellulose, and lignin, which are vital sources of monomeric carbohydrates that cause bioethanol formation through a fermentation process.[10,11] Cellulose provides structural support to plants, while hemicellulose and lignin are liable for strength and binding of all the components.[12]

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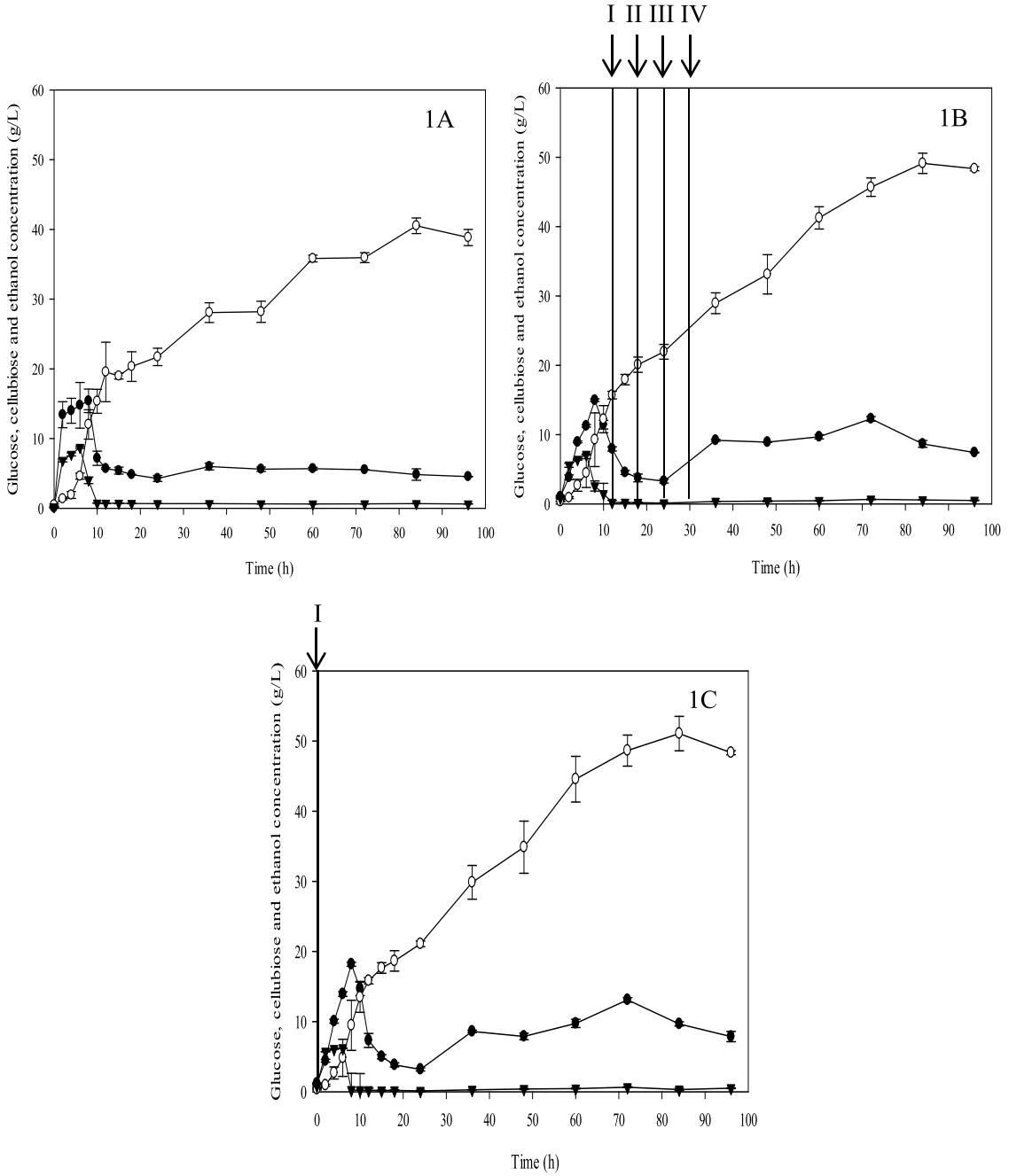

Figure 1. Effect of enzyme feeding strategy in fed-batch SSF. (A) No future enzymes were added. (B) Addition of the enzyme at four different times. (C) Addition of all enzymes at startup, 10% solid loading at the beginning, and 2.5% at 12, 18, 24, and 30 h with 20% (w/v) (solid circle) glucose, (upside down solid triangle) cellobiose, and (open circle) ethanol.

Pretreatment is an essential step in ethanol production.[13] OPT as a feedstock requires effective pretreatment prior to enzymatic hydrolysis, fermentation, and distillation. Pretreatment can partially remove lignin and hemicellulose and reduce cellulose crystallinity by enhancing the permeability of the porous matrix to improve enzymatic hydrolysis.[14,15]

After pretreatment of the lignocellulosic material, bioethanol can be produced through hydrolysis of cellulose and hemicellulose to pentose and/or hexose for ethanol production followed by fermentation.[16] The hydrolysis and fermentation steps can be executed as separate hydrolysis and fermentation (SHF) or together as simultaneous saccharification and fermentation (SSF). Moreover, SSF has numerous benefits over SHF including least enzyme requisition, higher product yield, less chances of foreign contamination, cost effectiveness, less inhibitory effects, and no product inhibition of β-glucosidase.[17]

In order to make a feasible strategy to substitute fossil fuel for the attainment of a higher ethanol concentration, it is compulsory to lessen the production cost, scale up ethanol production, and attain the highest possible ethanol concentration.[18] A cost-effective distillation process requires an ethanol concentration of higher than 4% (w/w), which implies a minimum of 8% (w/w) of fermentable sugars during enzyme hydrolysis.[19] Above and beyond, for a high glucose yield, it is necessary to perform the saccharification process with high

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Table 1. Fermentation Kinetic Parameters for Ethanol Production from an Oil Palm Trunk under Various Enzyme Feeding Strategies in the Flask[a]

state	ethanol concentration C_P (g/L)	ethanol yield $Y_{P/S}$ (g/g)	ethanol productivity Q_P (g/L·h)	ethanol theoretical yield (%)	maximum ethanol production hour
flask (300 mL total volume)					
fed-batch SSF, 20% (w/v)	$40.51^{c} \pm 1.10$	$0.24^{c} \pm 0.83$	$0.48^{c} \pm 0.19$	$47.00^{c} \pm 0.76$	84th
fed batch with enzyme and substrate feeding, 20% (w/v)	$49.12^{b} \pm 1.46$	$0.29^{ab} \pm 0.17$	$0.58^{a} \pm 0.66$	$57.00^{ab} \pm 0.67$	84th
fed-batch SSF with all enzymes at the start, 20% (w/v)	$51.05^{a} \pm 1.05$	$0.31^{a} \pm 0.92$	$0.61^{a} \pm 0.02$	$60.65^{a} \pm 0.32$	84th

[a]Note: [a,b]Means within the same column with different letters are significantly different ($P < 0.05$).

solid loading, which is more than 15% on dry weight basis.[20] However, using the SSF process at a high substrate loading has some shortcomings; for instance, a higher viscosity results in a lower efficiency of heat and mass transfer[21] and hinders enzyme substrate interaction. In addition, high substrate loading can produce inhibitors, especially glucose inhibition, which directly affects the ethanol yield and productivity.[19,22]

To overcome these problems, several modifications have been suggested for the SSF process including batch and fed-batch SSF approaches with the enzyme and substrate feeding strategy,[23] which promotes a rise in water-insoluble solids (WIS), producing a high conversion yield,[24] co-fermentation of xylose and glucose (SSCF),[25] hydrolysis followed by fed-batch SSF (PSSF),[26] fed batch, and horizontal bioreactor.[27] Hence, the strategy of a horizontal rotating bioreactor has shown promising results by effectively mixing the substrate and enzyme while using high solid loading for enzyme hydrolysis.[28] The strategy of PSSF includes a hydrolysis step with an optimum temperature provided to the enzyme followed by a lower-temperature SSF in a single batch, which allows SSF to overcome the drawbacks due to dissimilarity in the optimum temperature for the microbe and enzyme.[29] As follows, the greater enzymatic hydrolysis rate provided by PSSF causes effectual liquefaction and reduces the viscosity of the entire slurry.[30] For bioethanol production, SSF in a fermenter needs extensive trails.

In this study, the alkaline hydrogen peroxide (AHP) pretreated fibers produced in our previous study[31] were used for bioethanol production from a 300 mL flask to a 1 L agitating fermenter. This study is the first exploration for scaling up of bioethanol production from AHP pretreated OPT fibers. The effects of the enzyme feeding strategy, high substrate loading, and pre-hydrolysis step on SSF were assessed to scale up bioethanol production.

2. RESULTS AND DISCUSSION

2.1. Oil Palm Trunk Composition. The main components in the raw material oil palm trunk, exhibited by compositional analysis on dry weight (DW), are extractive substances (11.22%), hemicellulose (30.22%), cellulose (38.67%), lignin (23.76%), pentosan (23.30%), and ash (1.62%). Nonetheless, post- and pretreatments revealed that the content of cellulose enhanced to 73.96%, and there was a reduction in the percentage quantities of hemicellulose (12.9%), lignin (11.68%), pentosan (1.17%), and ash (0.95%). In accordance with the literature, the lignin content in OPT fibers was lower than those of apricot seed kernel (24.65%), apricot kernel shell (47.97%), Jatropha seed cake (25.0%), and black cumin seed cake (26.73%).[35] However, the 23.76% dry weight lignin content of this study corresponded well with the findings of Adela et al. and Choi et al.[36,37]

2.2. Effect of Enzyme Loading Investigated in High Solid Fed-Batch Simultaneous Saccharification and Fermentation (SSF) for Bioethanol Production. *2.2.1. Effect of Enzyme Feeding.* To increase the ethanol production, fed-batch SSF with an enzyme feeding strategy was adopted in three different manners. (I) No further enzymes were added in a high solid loading of 20% (w/v) AHP-treated fibers during the fed-batch SSF process. The concentrations of cellobiose, glucose, and ethanol in fermentation are given in Figure 1A. The cellobiose concentration rose quickly at the beginning but depleted after 6 h and remained constant after 12 h till the end of fermentation. The glucose concentration was found to be maximum after 6 h that lowered after 24 h but increased again after 36 h when fed with the substrate at 24 h, although it remained constant later. The corresponding ethanol concentration increased in the first 36 h and reached its uppermost concentration, i.e., 40.12 ± 1.46 g/L after 84 h of fermentation.

(II) Enzymes were added at four different time intervals (12, 18, 24, and 30 h) with 20% (w/v) solid loading. The glucose concentration rose at the commencement of fermentation but lessened after 8 h. Likewise, it rose again at 36 and 72 h, which might be due to enzyme feeding causing adsorption of enzymes to the substrate in a fermenter in comparison with the enzyme feeding and substrate separately. The ethanol production was continued till 84 h, which lessened later. The highest ethanol concentration of 49.12 ± 1.46 g/L was observed after 84 h (Figure 1B). The results of this study were found to be better than the findings of Triwahyuni,[38] who managed to produce an ethanol concentration of 47.83 g/L (10 FPU/g).

On the other hand, (III) the effect of all enzymes added at the startup of fed-batch SSF was investigated. The glucose quantity rose initially but kept on depleting after 8 h. Furthermore, the glucose concentration increased again at 36 and 72 h, which might be due to feeding of the substrate at 12, 18, 24, and 30 h.[39] Finally, the highest ethanol concentration obtained was 51. 05 ± 1.05 g/L after 84 h (Figure 1C). In addition, it clearly presented the vitality of stimulating the hydrolysis rate at first, in contrast with the steady enzyme feeding. Sequentially, there was more monomeric hexose (glucose) release from its polymer (cellulose).[40] The enzyme feeding strategy is helpful for its stability, but it can decrease the production of ethanol, pH, and temperature. Additionally, at a greater substrate loading, the synergistic effect of these factors can severely affect enzyme activity.[41] Moreover, Zhao et al.[42] also favored the addition of all enzymes at startup rather than at enzyme feeding. The binding of enzymes with compounds avoids deactivation of enzymes, which consequently stay in active form in the fermenter for a longer period of time.[43]

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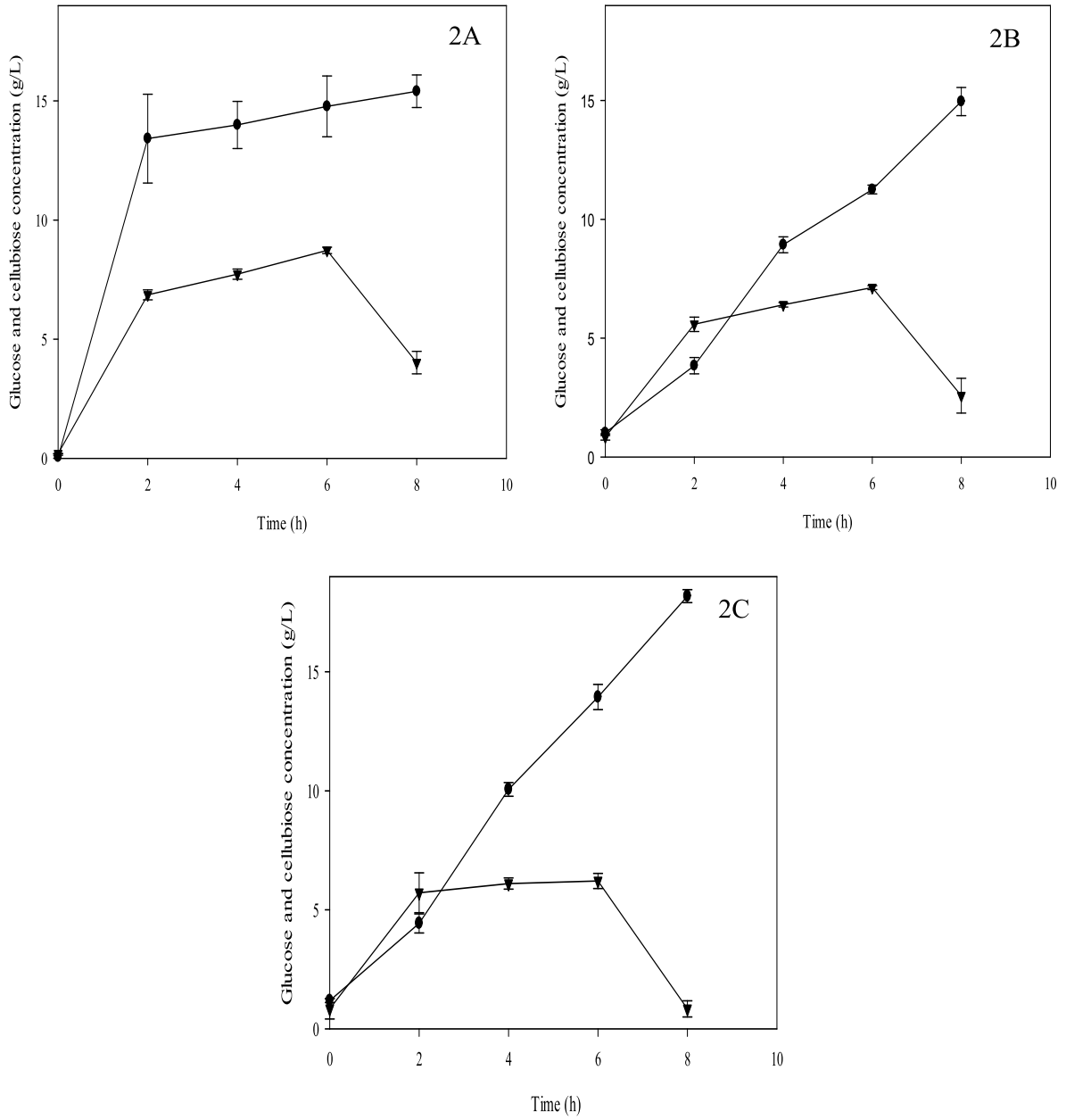

Figure 2. Effect of the enzyme feeding strategy on enzyme hydrolysis in the initial 8 h of fermentation in the flask. (A) No further enzymes were added. (B) Addition of enzymes at four different times. (C) Addition of all enzymes at the start-up of fed-batch SSF at 20% (w/v) solid loading of AHP-treated fibers (solid circle) glucose and (solid upside down triangle) cellobiose.

2.2.2. Fermentation Kinetics (Flask). Fed-batch SSF fermentation was extensively studied to produce ethanol from pretreated OPT fibers; the detailed kinetic parameters are shown in Table 1. Fed-batch SSF was performed with the addition of all enzymes at the start of fermentation. It resulted in an utmost ethanol concentration of 51. 05 ± 1.05 g/L after 84 h of fermentation, displaying 0.61 ± 0.02 g/L·h ethanol productivity, 0.31 ± 0.92 g/g ethanol yield, and 60.65% theoretical ethanol yield (Table 1). However, a significant ($p < 0.05$) increase in ethanol concentration, ethanol yield, and ethanol theoretical yield was observed when all enzymes were added at the start of fermentation. Furthermore, the maximum ethanol production hour remained the same (Table 1). The results were found to be satisfactory when compared with the finding of Sewsynker-Sukai and Kana,[44] who achieved 35.04 g/L bioethanol concentration with a relatively high enzyme loading of 30 FPU/g. In comparison with enzyme concentration, enzyme feeding at different intervals and enzyme loading at the start of fed-batch SSF relatively gave a higher ethanol concentration, ethanol productivity, ethanol yield, and ethanol theoretical yield.

2.2.3. Enzyme Hydrolysis of the Initial 8 h. In the current study, enzyme hydrolysis of the initial 8 h was studied to observe the effect of the enzyme feeding strategy. After 8 h of enzyme hydrolysis, the feeding strategy with (I) and (II) gave glucose concentrations of 15.40 and 15.15 g/L, respectively (Figure 2A,B). Moreover, the enzyme feeding strategy with (III) significantly ($p < 0.05$) improved the glucose concentration to 18.17 g/L after 8 h (Figure 2C). Furthermore, the initial velocity (V_0) of enzymes was calculated using the Michaelis−Menten equation in the beginning of fed-batch SSF (0 to 8 h). The enzyme feeding

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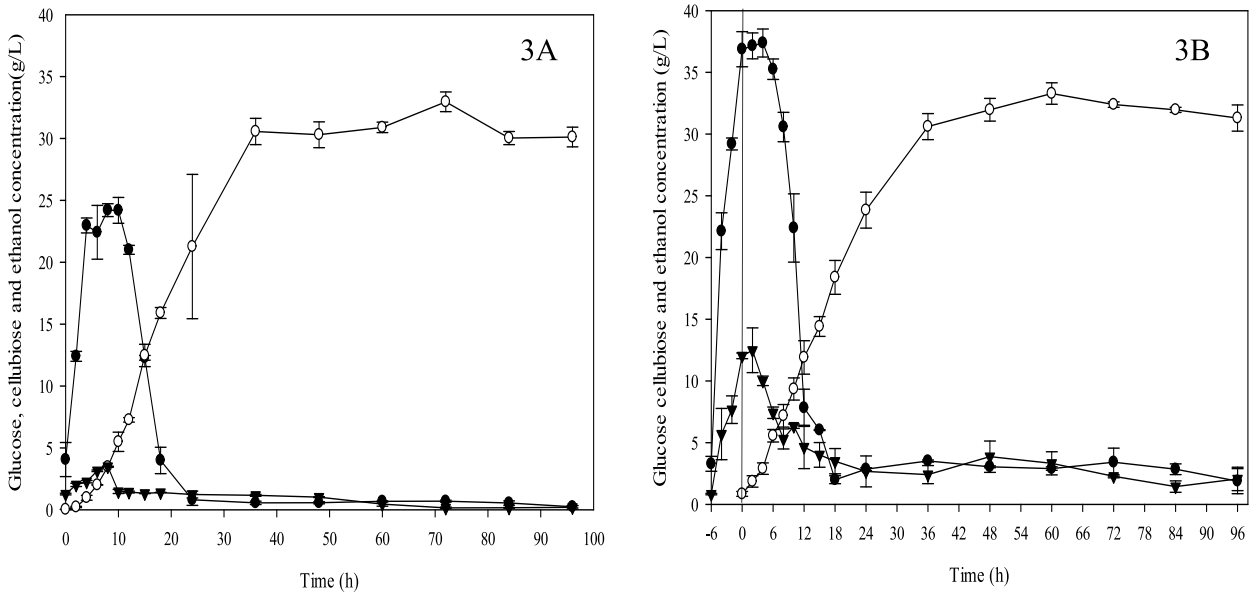

Figure 3. Comparison of (A) simultaneous saccharification and fermentation (SSF) and (B) pre-hydrolysis simultaneous saccharification and fermentation (PSSF) in the fermenter with 5% (w/v) of initial solid loading, addition of 2.5% of the substrate at 6 and 12 h with a total solid loading of 10% (w/v) (solid circle) glucose, (solid upside down triangle) cellobiose, and (open circle) ethanol.

strategy includes (I) no further addition of the enzyme, which exhibited an initial velocity (V_0) of 1.92 g/h, and (II) addition of the enzyme at four different times, which gave an initial velocity of 1.89 g/h. Interestingly, when all enzymes were added in the startup of fermentation (III), a substantial increase was observed in the initial velocity of enzymes (V_0), i.e., 2.27 g/h. On the basis of the current results, it was decided to step-wise-produce ethanol from a 500 mL shaken flask to a 1 L fermenter. A balanced fed-batch SSF was studied by developing suitable enzyme and substrate feeding strategies. Moreover, pre-hydrolysis simultaneous saccharification and fermentation (PSSF) along with multifeeding of the enzymes and substrates was also studied to improve the ethanol production.

2.3. Effect of Pre-hydrolysis SSF for Ethanol Production in a 1 L Fermenter. *2.3.1. Substrate Feeding Strategy in the Fermenter.* To increase the ethanol yield, productivity, and proper agitation, a substrate feeding strategy was used. The initial substrate loading was 5% (w/v), whereas the remaining 2.5% (w/v) substrate was added at 6 and 12 h, respectively, which may overcome the problem of poor agitation and homogeneity of the substrate as the fibers were constantly broken down, resulting in the reduction of fermentation viscosity. Figure 3A shows that the glucose concentration increased at the start but decreased after 12 h, whereas the cellobiose concentration slightly increased at the start but decreased after 8 h. The cellulase enzymes include endoglucanase, exoglucanase (cellobiohydrolase), and β-glucosidase, which act synergistically. The endoglucanase generates reducing and nonreducing chain ends for cellobiohydrolase that releases cellobiose (dimer), which is converted to glucose by β-glucosidase.[45] As Cellic CTec2 has high cellulase and β-glucosidase activities, it produces an elevated level of glucose. Cellic CTec2 was reported for efficient hydrolysis of hydrothermally pretreated wheat straw.[46] This higher efficiency is probably due to both the overall cellulase and β-glucosidase activities.[47]

The ethanol formation initiated after 6 h increased rapidly in the first 36 h but dropped at 72 h of fermentation, giving the highest concentration of ethanol at 31.77 ± 0.84 g/L. The SSF in fed-batch mode may overcome such a substrate feeding strategy that helps yeast to turn higher inhibitory compounds into the compounds with low inhibition potential, causing detoxification of the fermentation medium.[48] The inclusive operating duration can be reduced through a multifeeding technique. Accordingly, from the industrial standpoint, fed-batch SSF is an ideal strategy so far.[24]

In addition, the effect of pre-hydrolysis in SSF for ethanol production was also investigated in a 1 L fermenter. The pre-hydrolysis step of 6 h sufficiently increased the liquid in the slurry, which helped in the release of hexose (glucose). The highest glucose concentration (36.81 ± 1.42 g/L) prior to the addition of *S. cerevisiae* SC90 was obtained with the pre-hydrolysis step. Glucose as the end product of cellulose hydrolysis is released by β-glucosidase through eliminating the effect of cellobiose inhibition on endoglucanase and cellobiohydrolase, consequently letting more efficient cellulolytic enzyme activity.[49] The overall fermentation was swift and initiated as soon as *S. cerevisiae* SC90 was added to the reactor. The maximum ethanol was observed in the first 36 h of fermentation; however, a reduction in the ethanol quantity rate occurred owing to the rapid consumption of *S. cerevisiae* SC90. The presence of enzymes in the fermentation medium caused a gradual and steady hydrolysis of the substrate on account of a suboptimal temperature (40 °C), which may ascend the ethanol formation with thorough utilization of initial glucose. The highest ethanol concentration observed after 60 h was 33.28 ± 0.31 g/L (Figure 3B).

2.3.2. Fermentation Kinetics (Fermenter). To improve ethanol production, the substrate feeding strategy was used in a 1 L fermenter. It was observed that the substrate feeding strategy significantly improved mixing of the substrate, reduced fermentation time, and energy consumption for mixing,[50] which are the most important steps for the production of ethanol at a larger scale.[22] The highest ethanol concentration

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Table 2. Fermentation Kinetic Parameters for Ethanol Production from an Oil Palm Trunk in a 1 L Fermenter Using SSF and PSSF Processes[a]

state	ethanol concentration C_P (g/L)	ethanol yield $Y_{P/S}$ (g/g)	ethanol productivity Q_P (g/L·h)	ethanol theoretical yield (%)	maximum ethanol production hour
fermenter (1 L volume)					
fed-batch SSF (feeding at 0, 6, and 12 h) 10% (w/v)	31.77[a] ± 0.84	0.38[a] ± 0.12	0.44[b] ± 0.18	74.36[b] ± 2.01	72th
PSSF (feeding at 0, 6, and 12 h) 10% (w/v)	33.28[a] ± 0.31	0.40[a] ± 0.18	0.56[a] ± 0.18	78.28[a] ± 0.45	60th

[a]Note: [a,b]Means within the same column with different letters are significantly different ($P < 0.05$).

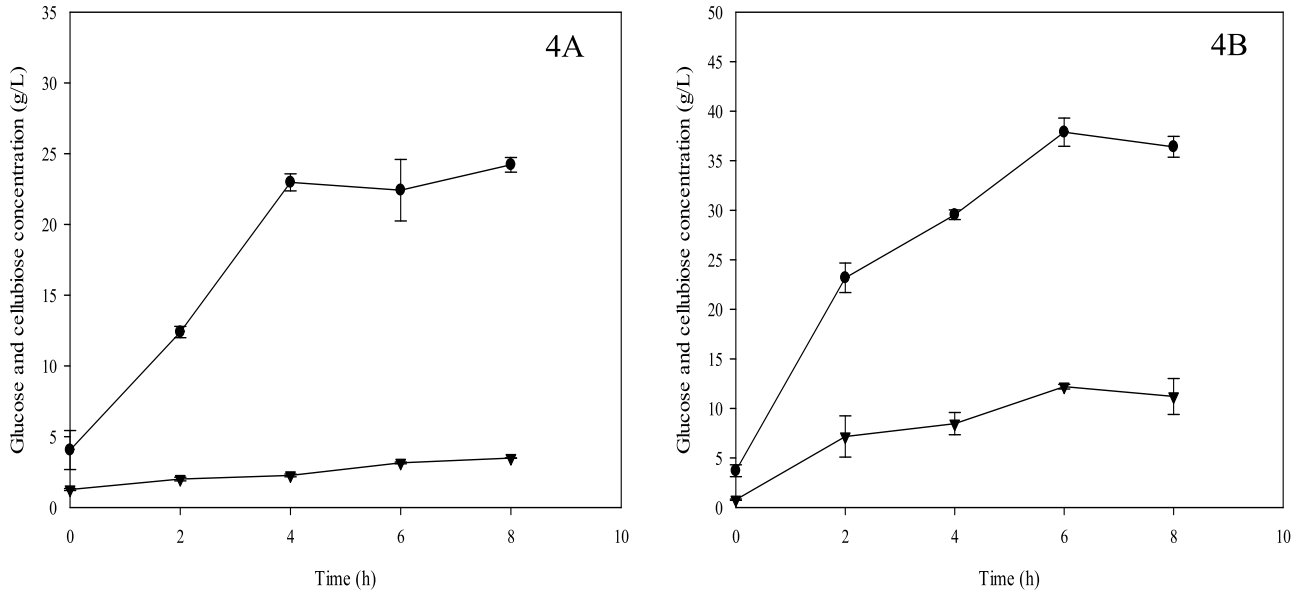

Figure 4. Effect of enzymes on enzyme hydrolysis in the initial 8 h of fermentation. (A) Simultaneous saccharification and fermentation (SSF) and (B) pre-hydrolysis simultaneous saccharification and fermentation (PSSF) in a fermenter with 5% (w/v) of the initial solid loading (solid circle) glucose and (solid upside down triangle) cellobiose.

of 31.77 ± 0.84 g/L was displayed after 72 h with an ethanol productivity of 0.44 ± 0.18 g/L·h, ethanol yield of 0.38 ± 0.12 g/g, and ethanol theoretical yield of 74.36% (Table 2). In comparison, pre-hydrolysis simultaneous saccharification and fermentation (PSSF) was done with fed-batch SSF at 50 °C for 6 h with 5% (w/v) of the initial substrate. With the addition of yeast, the temperature of the fermenter was reduced to 40 °C, and the remaining 2.5% of the substrate was added at 6 and 12 h of fermentation. It was clearly observed that the pre-hydrolysis step in consort with feeding improved the homogeneity and mixing process, which resulted in a reduction of the overall fermentation time.[51]

Fed-batch PSSF with 10% (w/v) substrate increased ethanol concentration to 33.28 ± 0.31 g/L after 60 h with attainment of 0.56 ± 0.18 g/L·h ethanol production, 0.40 ± 0.18 g/g ethanol yield, and 78.28% theoretical ethanol yield. However, a significant ($p < 0.05$) difference between ethanol productivity and ethanol theoretical yield was observed. Nonetheless, the statistical value analysis indicated no significant ($p < 0.05$) differences between ethanol concentrations and ethanol yields (Table 2). The findings of the current study were better compared with that of Dimos et al.,[52] who managed to produce an ethanol concentration of 32.32 g/L with 15% (w/v) substrate loading after 6 h of pre-hydrolysis. Interestingly, various studies have reported less effect of the pre-hydrolysis step before fermentation on ethanol formation,[53] although Kalogiannis et al.[54] observed a significant increase in the ethanol yield while using the pre-hydrolysis step with fermentation. The obtained results suggested that the effect of pre-hydrolysis on ethanol yield was small, but it resulted in a faster liquefaction and low viscosity of the slurry that helped reduce energy cost (less stirring power) and overall fermentation time.

2.3.3. Enzyme Hydrolysis of the Initial 8 h. The enzyme hydrolysis of the initial 8 h was studied to observe the effect of enzyme on substrate feeding strategy in SSF and PSSF in the fermenter. The feeding strategy in SSF gave a glucose concentration of 24.21 g/L (Figure 4A), while the initial velocity (V_0) of the enzyme (0 to 8 h) was 3.02 g/h. In comparison to SSF, PSSF significantly ($p < 0.05$) improved the glucose concentration and initial velocity (V_0) of enzymes during the initial 8 h of enzyme hydrolysis. The PSSF with 10% (w/v) of the substrate exhibited a glucose concentration of 37.14 g/L (Figure 4B), while the initial velocity (V_0) of the enzyme was observed to be 4.64 g/h. The results were found to be better than the findings of Zhu et al.,[55] who could manage to improve 11−16% of fermentable sugar yields from wheat straw by the pre-hydrolysis step in the initial hours of fermentation.

2.4. Effect of High Solid Loading of PSSF for Ethanol Production in a 1 L Fermenter. To upsurge the ethanol production for economical distillation, a higher solid loading of 20% (w/v) was used in PSSF. The pre-hydrolysis duration was fixed for 6 h with a 5% (w/v) substrate. The 2.5% (w/v)

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substrate was added at 0, 6, 12, 18, 24, and 30 h of fermentation. At the end of pre-hydrolysis, the medium contained 44.46 ± 0.69 g/L glucose. Once the temperature was lowered to 40 °C, the yeast *S. cerevisiae* SC90 was added to the fermenter, which rapidly started producing ethanol (Figure 5). Additionally, the increased glucose yield using Cellic

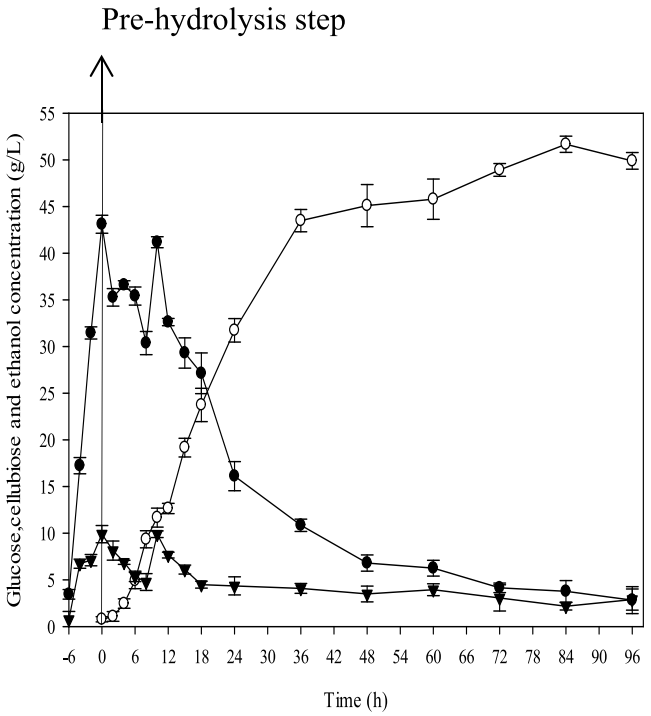

Figure 5. Effect of high solid loading in the pre-hydrolysis simultaneous saccharification and fermentation (PSSF) with 5% (w/v) of initial substrate loading, addition of 2.5% of substrate at 0, 6, 12, 18, 24, and 30 h with a total solid loading of 20% (w/v) (solid circle) glucose, (solid upside down triangle) cellobiose, and (open circle) ethanol.

CTec2 can be explained through its enhanced cellobiohydrolase activity.[56] The initial concentration of glucose steadily decreased with the passage of time, although a slight increase in glucose and cellobiose concentrations was observed after 8 h of fermentation, but it almost approached to the lowest point after 36 h, which may be attributed to the synergic interaction between endoglucanase and cellobiohydrolase, one of the most important factors in enzymatic hydrolysis of cellulose.[56] Endoglucanase exposes the polymer to cellobiohydrolase by randomly attacking the amorphous sites of cellulose.[57] Cellobiose and short oligosaccharides inhibit the activity of endoglucanase and cellobiohydrolase, whereas β-glucosidase is accountable for the rate-limiting step of the entire cellulolytic process and glucose production.[58] Until 36 h of PSSF, the ethanol quantity increased; conversely, it later decreased owing to enough substrate presence for exponential growth of SC90. Moreover, glucose was also utilized rapidly,[59] and the highest ethanol production of 51.66 ± 1.10 g /L (Figure 5) was observed after 72 h. Although the same ethanol concentration was seen, yet in the late hours of fermentation, when the fed-batch SSF was performed in a flask without pre-hydrolysis step. An ethanol concentration of 51.66 g/L favors the distillation process as an ethanol concentration higher than 4% (w/v), i.e., 40 g/L in the fermentation broth is a benchmark for efficient distillation, considering the energy consumption and efficiency of the ethanol recovery process.[60−62] The pre-hydrolysis caused faster glucose consumption, in comparison with the process without it, which in turn produced a higher ethanol concentration in lesser time.[63] Reduction in fermentation time could be attributed to the monomeric carbohydrates at the commencement of the fermentation that boosted the stimulation of *S. cerevisiae* resulting in the reduced fermentation time.[64]

2.4.1. Fermentation Kinetics (Fermenter). To reduce the exertion of the adverse influence triggered by a high solid loading system to produce high ethanol by *S. cerevisiae* SC90, a fed-batch system was carried out with the pre-hydrolysis step to determine a suitable condition. It was observed that fed-batch PSSF improved the sugar and ethanol concentrations and reduced the overall fermentation time. The highest high ethanol concentration of 51.66 ± 0.84 g/L was observed after 72 h of fermentation with an ethanol production of 0.31 ± 0.32 g/L·h, ethanol yield of 0.72 ± 0.24 g/g, and ethanol theoretical yield of 60.66% (Table 3). In comparison to fed-batch PSSF with 10% (w/v), the PSSF with 20% (w/v) gave no significant ($p < 0.05$) differences among the ethanol yield values, and a slight decrease in the ethanol theoretical yield and increase in the fermentation time were observed. In addition, PSSF with 20% (w/v) significantly ($p < 0.05$) improved the ethanol yield from 0.56 ± 0.18 to 0.72 ± 0.24 g/g (Table 3). The pre-hydrolysis step in SSF has the advantage of SHF that allows the enzyme and yeast to work at their optimum conditions. In contrast, the results were found to be higher than those by Lu et al.,[65] who attained 49.5 g/L with 30% solid loading, whereas Sewsynker-Sukai and Kana and Gao et al.[44,66] used 25 and 17.50% solid loading and achieved only 36.25 and 36.92 g/L ethanol, respectively.

2.4.2. Enzyme Hydrolysis of the Initial 8 h. During PSSF, a significant ($p < 0.05$) increase in the glucose concentration was observed in the initial 8 h of enzyme hydrolysis. PSSF after 8 h of enzyme hydrolysis with both (I) 10% (w/v) and (II) 20% (w/v) of substrate loading gave a glucose concentration of 37.14 and 35.27 g/L, respectively (Figures 4B and 6). The initial velocity (V_0) of enzymes in PSSF significantly ($p < 0.05$) improved during 0 to 8 h of enzyme hydrolysis with both (I) 10% (w/v) and (II) 20% (w/v) of substrate loading. The

Table 3. Fermentation Kinetic Parameters of Ethanol Production from High Solid Loading Using the PSSF Process[a]

state	ethanol concentration C_P (g/L)	ethanol yield $Y_{P/S}$ (g/g)	ethanol productivity Q_P (g/L·h)	ethanol theoretical yield (%)	maximum ethanol production hour
fermenter (1 L volume)					
PSSF (feeding at 0, 6, and 12 h) 10% (w/v)	33.28^{b} ± 0.31	0.40^{a} ± 0.18	0.56^{b} ± 0.18	78.28^{a} ± 1.31	60th
PSSF (feeding at 0, 6, 12, 18, 24, and 30 h) 20% (w/v)	51.66^{a} ± 0.84	0.31^{ab} ± 0.32	0.72^{a} ± 0.24	60.66^{b} ± 0.71	72th

[a]Note: [a,b]Means within the same column with different letters are significantly different ($P < 0.05$).

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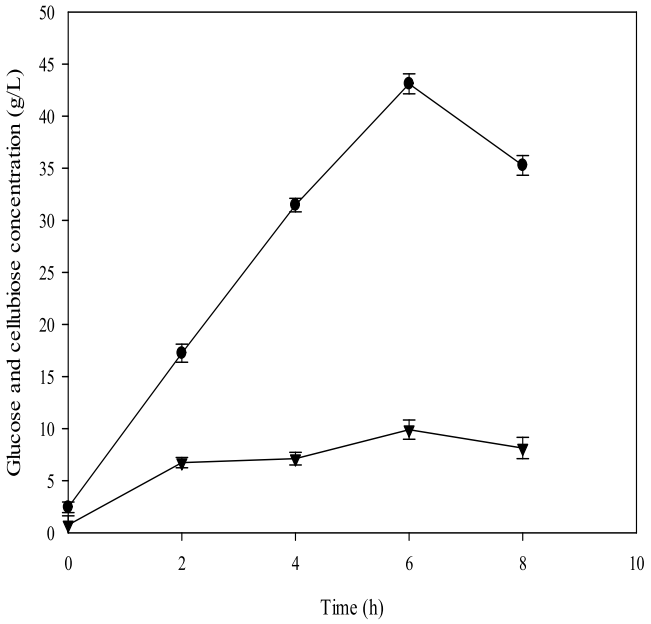

Figure 6. Effect of the enzyme feeding strategy on enzyme hydrolysis in the initial 8 h of PSSF in a fermenter (solid circle) glucose and (solid upside down triangle) cellobiose.

initial velocity (V_0) of enzymes with both (I) 10% (w/v) and (II) 20% (w/v) exhibited 4.64 and 4.40 g/h, respectively.

3. CONCLUSIONS

The aim of finding the eccentric ways of energy generation that are sustainable, renewable, low cost, and ecofriendly to replace conventional fossil fuels is the order of the day. The oil palm trunk, which is rich in carbohydrates, has the efficacy of being an alternative raw material for bioethanol production. With the objective of producing an efficient amount of ethanol and placing lignocellulosic ethanol into the market, the AHP pretreated OPT fibers were used for simultaneous saccharification and fermentation (SSF). The SSF process is preferable due to lesser enzyme requisition, lower operational costs, and improved productivity. Pre-hydrolysis has been shown to diminish the viscosity of the material. Moreover, the pre-hydrolsysis step along with the substrate feeding step improved the mixing efficiency and homogeneity of high solid loading and enzymes, which increased the ethanol yield and reduced the overall fermentation time. The pre-hydrolysis prior to SSF fermentation significantly increased initial velocity (V_0) of enzymes and provided the optimum conditions for both the enzyme and yeast to utilize the substrate sufficiently for efficient ethanol production. It was concluded that the pre-hydrolysis step with fed-batch SSF can effectually upsurge the solid loading to acquire elevated ethanol production, i.e., 51.66 g/L, which can finally reduce the production cost and favors the next step in the process that is distillation.

4. MATERIALS AND METHODS

4.1. Materials. The oil palm trunk (*Elaeis guineensis* Jacq.) was bought from a local agriculturist in Plai Phraya District, Krabi Province, Thailand. All chemical reagents were analytical grade and purchased from Sigma-Aldrich. The following were used: cellobiose (99%), ethanol (95%), glucose (99%), sodium hydroxide (≥97.0%), sulfuric acid (99%), yeast extract−peptone−dextrose (YPD) medium, Cellic CTec2 Novozymes, Erlenmeyer flask, steam explosion machine with a 2.5 L tank (Kumakai Nitto, Japan), rotary shaker (Infors HT Ecotron, USA), and 3 L glass fermenter ez2-Control (Applikon Biotechnology, USA).

4.2. Methods. *4.2.1. Pretreatment: Steam Explosion, Hot Water Washing, and Alkaline Hydrogen Peroxide Treatment.* Before initiating the pretreatment process, a wood chipper was used to chop OPT into 20 × 20 × 5 mm^3 chips and dried in the sunlight, and 150 g chips were subjected to steam explosion for 4 min, at 210 °C, in a 2.5 L tank.[32] The steam-exploded fibers were obtained by draining off and squeezing the slurry. The fibers were washed with hot water for 30 min with a solid:liquid ratio of 1:8 (g/mL) at 80 °C. In addition, tap water washing was done until the pH of the fibers was neutralized and hemicellulose removal was attained. An alkaline hydrogen peroxide treatment (AHP) with the conditions of 70 °C, 30 min and 3% H_2O_2 g /g of biomass[31] was used to remove lignin from OPT fibers. The standard analysis of the technical association of the pulp and paper industry (TAPPI) was used for compositional analysis of AHP-treated fibers: T264 om-97, (1997) for moisture content; T204 om-97 for extractives; T222 om-98 for acid insoluble lignin; T223 om-84 for pentosan (TAPPI, 1983d); and TAPPI T211 om-85 (TAPPI, 1983b) for ash. The α-cellulose was determined by TAPPI T203 om-93 (TAPPI, 1983c). The holocellulose was analyzed by the acid−chloride method of Browning. The laboratory analytical procedure (LAP) of the National Renewable Energy Laboratory (NREL)[33] was applied for measuring the cellulase activity of Cellic CTec2, expressed in filter paper unit (FPU); the cellulase activity of Cellic CTec2 was 178.5 FPU.

4.2.2. Starter Culture/Inoculum Preparation. Industrial yeast strain *Saccharomyces cerevisiae* SC90 was purchased from Liquor Distillery Organization Excise Department, Thailand. Starter culture was prepared from a stock culture using the streak plate technique on agar plates of (YPD): yeast extract (10 g/L), peptone (20 g/L), glucose (20 g/L), and agar (20 g/L) at 30 °C for 48 h. A single, marginal colony was picked by a loop and inoculated into an Erlenmeyer flask with 30 mL of liquid YPD media and incubated at 30 °C in a rotary shaker at 150 rpm. A culture of 18 h of incubation was used as the starting inoculum for fermentation.

4.2.3. Effect of Enzyme Loading Investigated in High Solid Fed-Batch Simultaneous Saccharification and Fermentation (SSF) for Bioethanol Production. The experimental procedure of the fed-batch SSF was held in an Erlenmeyer flask (500 mL) carrying 300 mL of 20% AHP-treated fibers, YP medium, 10% starter culture, and 10 FPU/g of Cellic CTec2 at 40 °C for 96 h and 150 rpm. Ten percent of solid loading was added at the beginning and 2.5% at 12, 18, 24, and 30 h with 20% (w/v) solid loading. Three different sets of the experiments were performed in three replicates, each on the basis of enzyme addition as: (I) no further addition of the enzyme, (II) addition of enzyme at four different times, and (III) addition of the enzyme at the startup of fermentation.

4.2.4. Effect of Pre-hydrolysis SSF for Ethanol Production in a 1 L Fermenter. To produce ethanol from a 300 mL flask to a 1 L agitating fermenter, a similar SSF condition was performed with the substrate feeding strategy. A 3 L fermenter containing 1 L of fermentation media, composed of 10% fibers and YP medium, buffered with 50 mM sodium citrate (pH 4.8), and autoclaved for sterilization at 121 °C for 15 min. Ten percent inoculum and 10 FPU g/L of enzymes were poured

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together in the fermenter. A similar fed-batch SSF was performed in the fermenter for the incubation time of 96 h at 40 °C with agitation of 150 rpm. Triplicate experiments were performed with the addition of all enzymes at the start, solid loading of 5% (w/v) at the beginning, and 2.5% fibers at 6 and 12 h.

The impact of pre-hydrolysis and simultaneous saccharification and fermentation (PSSF) was also studied. The AHP-treated fibers were hydrolyzed through an enzyme mixture at 50 °C for 6 h. Later on, the suspension temperature was lowered to 40 °C for adding the *S. cerevisiae* SC90 inoculum. The initial solid loading was 10% (w/v) with all enzymes added at the startup of SSF. The experiments were performed in three replicates.

4.2.5. Effect of High Solid Loading of PSSF for Ethanol Production in a 1 L Fermenter. The effect of high solid loading of PSSF for ethanol production was investigated. The pre-hydrolysis step before SSF fermentation was performed in a fermenter with 1 L working volume. The experimental methods were operated with 10 and 20% (w/v) high solid loading and compared with each other to study the effect of high solid loading in PSSF. The addition of 5% solid loading was done at the beginning and 2.5% at 0, 6, 12, 24, and 30 h with a total of 20% (w/v) solid loading. The experiments were performed in triplicate, and average values along with the standard deviations were also reported.

4.3. Analytical Methods. *4.3.1. Analysis of Cellobiose, Glucose, and Ethanol by HPLC.* High-performance liquid chromatography (HPLC) (Agilent Technologies, Germany) using an Aminex HPX-87H column (Bio-Rad, USA) and refractive index detector (RID) was used to analyze the cellobiose, glucose, and ethanol concentrations. 50 mM H_2SO_4 at a flow rate of 0.6 mL min^{-1} maintained at 50 °C was used as the mobile phase.[34]

4.3.2. Fermentation Kinetics for Ethanol Production. Kinetic parameters for SSF were calculated in accordance with (NREL, 2008) the following equations:

ethanol productivity (Q_P)

$$Q_p\ (\mathrm{g/L{\cdot}h}) = \frac{P_0 - P_t}{t - t_0} \tag{1}$$

ethanol yield $Y_{P/S}$ (g/g)

$$Y_{P/S}\ (\mathrm{g/g}) = \frac{[P_t \times P_0]}{f\ [\mathrm{Biomass}]1.11} \tag{2}$$

theoretical ethanol yield (%)

$$\text{theoretical ethanol yield} = \frac{[P_t - P_0]}{0.51\, f[\mathrm{Biomass}]1.11} \times 100 \tag{3}$$

where in eq 1, P_0 and P_t show the ethanol concentrations at the beginning (time = t_0) and at the end of the ethanol maximum (time = t). The ethanol yield ($Y_{P/S}$) in eq 2 was calculated from the initial and final concentrations of ethanol, where f represents the cellulose fraction (dry weight) of the biomass. The biomass fiber (g/L) at the beginning and 1.11 indicates theoretical conversion of cellobiose to glucose. The theoretical ethanol yield (%) in eq 3 was measured from the initial and final concentrations of ethanol, whereas 0.51 is the theoretical conversion of glucose to ethanol.[32]

The initial velocity of enzymes from (0 to 8 h) was calculated using the equation given below

initial velocity of enzymes

$$V0 = \frac{V_{max}[S]}{K_m + [S]} \tag{4}$$

where in eq 4, V_0 represents the initial velocity of enzymes, V_{max} is the maximum velocity at the initial concentration of the substrate (mol/min), [S] represents the substrate concentration, and K_m shows the substrate at half V_{max} (mol/L).

4.4. Statistical Analysis. The data was expressed as the mean values and standard deviation (SD). The significant difference between ethanol concentrations (g/L), ethanol productivity (g/L·h), ethanol yield (g/g), and theoretical ethanol yield (%) were analyzed by the Duncan new multiple range test, using SPSS (24 version IBM Corp, USA), and the level of significance was adjusted at 95% ($p < 0.05$).

AUTHOR INFORMATION

Corresponding Author

Pramuk Parakulsuksatid − *Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand*; Phone: +66 664 45945; Email: fagipmp@ku.ac.th; Fax: +66 256 25046

Authors

Afrasiab Khan Tareen − *Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand*; orcid.org/0000-0001-5761-4044

Vittaya Punsuvon − *Department of Chemistry, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*

Imrana Niaz Sultan − *Department of Biotechnology, Faculty of Life Sciences and Informatics, BUITEMS, Quetta 87300, Pakistan*

Muhammad Waseem Khan − *Department of Biotechnology, Faculty of Life Sciences and Informatics, BUITEMS, Quetta 87300, Pakistan*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.1c03111

Notes

The authors declare no competing financial interest.

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