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Research article

Improved bioethanol production from oil palm empty fruit bunch using different fermentation strategies

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Abstract

Importance of the work: Bioethanol production from oil palm empty fruit bunch (OPEFB) may be improved by changing the fermentation strategy.

Objectives: To investigate different fermentation strategies to achieve the highest bioethanol concentration and the lowest overall process time.

Materials & Methods: For the first strategy, OPEFB was pretreated, hydrolyzed and fermented based on separate hydrolysis and fermentation (SHF) with saccharification at 50°C and fermentation at 30°C. The second strategy involved simultaneous saccharification and fermentation (SSF), with both at 37°C. The third strategy applied delayed simultaneous saccharification and fermentation (DSSF), involving hydrolyzation at 50°C that was subsequently reduced to 37°C, after which the yeast was inoculated and the processing continued. The bioethanol concentrations and overall process times of the three strategies were compared.

Results: OPEFB consisted of 43.3 ± 0.89% cellulose, 20.3 ± 0.67% hemicellulose and 13.8 ± 0.65% lignin. After NaOH pretreatment, the cellulose content increased to 72.1 ± 0.70%. Different enzyme loadings (Cellic® CTec2) were used at 5 filter paper units (FPU)/g, 10 FPU/g and 15 FPU/g of substrate. Reducing sugar was produced corresponding to the enzyme loading. However, 10 FPU was chosen as the optimum, with glucose being about 80% of the attained reducing sugar. Hence, it was appropriate for *Saccharomyces cerevisiae* fermentation. Then, the bioethanol production was compared for the three different fermentation strategies (SHF, SSF, and DSSF). The highest bioethanol production and productivity were from DSSF at 26.1 ± 0.18 g/L and 0.36 g/L/hr, respectively ($p < 0.05$). In addition, DSSF had the shortest overall process time of 73 hr.

Main finding: The DSSF strategy was the best for bioethanol production from OPEFB producing the highest bioethanol concentration of 26.1 ± 0.18 g/L ($p < 0.05$) and the shortest process time of 73 hr compared to SHF and SSF.

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Introduction

During the last decade, one of the critical threats to the world has been the depletion of fossil resources in combination with an increase in energy consumption, which has resulted in a dilemma between the essential need for fuel and food while searching for sustainable non-edible feedstock for biofuel production (Derman et al., 2018). Therefore, second-generation feedstock as non-edible biomass for bioethanol production is being generally replaced by agricultural or lignocellulosic biomass (Nurfahmi et al., 2019; Kirdponpattara et al., 2022). Hence, studies have investigated the production of lignocellulosic bioethanol from cheap renewable, low-cost sources (Khomlaem et al., 2023) such as wheat straw (Qiu et al., 2018), sweet potato peel (Mithra et al., 2018), rice straw (Todhanakasem et al., 2019; Singh et al., 2020;), waste bamboo (Song et al., 2020), corn cob (David et al., 2020), sugarcane bagasse (Saha et al., 2019) and sweet sorghum bagasse (Thanapimmetha et al., 2019).

According to estimates, the annual global yield of oil palm empty fruit bunch (OPEFB) is approximately 76 million t (Yimlamai et al., 2021). OPEFB comprises 20.4–32.5% lignin, 25.3–33.8% hemicellulose and 37.3–46.5% cellulose (Mardawati et al., 2022). Recently, OPEFB has been considered a suitable substrate for bioethanol due to its high cellulose content (Sukhang et al., 2020; Mardawati et al., 2022). However, due to the resistance to enzymatic hydrolysis and its complex structure, lignocellulosic biomass cannot be used directly for fermentation (Khomlaem et al., 2023). Instead, it needs to be pretreated and hydrolyzed before fermentation.

Consequently, the aims of the current study were to investigate the chemical pretreatment of OPEFB and to compare various enzymatic hydrolysis and fermentation options of the treated OPEFB for bioethanol production. The three fermentation strategies investigated were: separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) and delayed simultaneous saccharification and fermentation (DSSF). In addition, the study aimed to evaluate bioethanol production by *Saccharomyces cerevisiae* TISTR 5606 which is frequently used in several industrial processes to produce bioethanol. Finally, the overall bioethanol processing time of the three fermentation strategies was compared and discussed.

Materials and Methods

Materials

The OPEFB used as a substrate in this study was obtained from Suksomboon Vegetable Oil Co., Ltd., Nong Yai, Chonburi, Thailand. The OPEFB sample was washed and sun-dried before storing in dry containers. Next, it was cut and sieved into 1–2 mm particles. The composition of the untreated OPEFB was analyzed according to the procedure of Goering and Soest (1970). The percentages of cellulose, hemicellulose, lignin and ash were determined using neutral detergent fiber and acid detergent fiber solutions. The overall bioethanol production from OPEFB is schematically represented in Fig. 1.

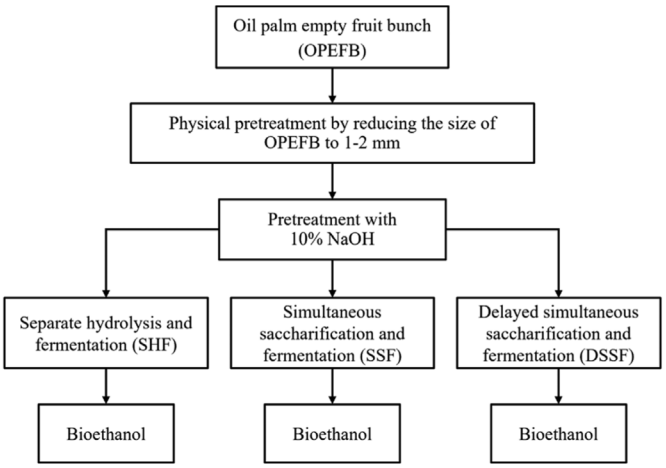

Fig. 1 Schematic representation of bioethanol production from oil palm empty fruit bunch (OPEFB)

The Cellic® CTec2 enzymes (Novozymes; Denmark) were purchased from Brenntag Ingredients (Thailand) Public Co., Ltd. The enzyme activity was tested using a filter paper assay of 275 filter paper units (FPU)/mL (Ghose, 1987). The enzyme hydrolysis conditions were pH at 5.0–5.5 and temperature at 45–50°C (Novozymes, 2010).

Yeast and medium preparation

S. cerevisiae TISTR 5606, purchased from the TISTR culture collection in Thailand, was evaluated for its ability to produce bioethanol using OPEFB as a substrate. The yeast was plated on yeast peptone dextrose (YPD) agar and stored at 4°C. The microbial culture was prepared as single yeast colonies in a liquid medium and incubated at 30°C for 12 hr.

The YPD medium composition containing 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose (Tareen et al., 2021) was used for experiments. In addition, the YPD medium without glucose was added to the OPEFB as a supplement.

Oil palm empty fruit bunch pretreatment

NaOH solution (10% weight per volume, w/v) was used to treat the OPEFB sample. Dry OPEFB (10 g/100 mL) and 10% (w/v) NaOH solution were placed in an autoclave for 1 hr at 121°C and 103.4 kPa, according to Thanapimmetha et al. (2019). After the solid residue had been recovered and cooled to room temperature, it was neutralized by washing with distilled water. The recovered solid residue was dried for 48 hr at 65°C to a constant weight. The chemical components of the treated OPEFB were analyzed according to the methods of Goering and Soest (1970).

Enzymatic hydrolysis

Enzymatic hydrolysis was performed for 96 hr at 50°C and 140 rpm in a shaking incubator using 250 mL Erlenmeyer flasks containing 100 mL of working volume. The substrate concentration was 10 g/100 mL (50 mM sodium citrate buffer at pH 5.0). The enzyme loadings (Cellic® CTec2) were 5 FPU/g, 10 FPU/g and 15 FPU/g substrate. The 3,5-dinitro salicylic acid (DNS) technique was used to determine the amount of reducing sugar in samples collected every 24 hr (Khomlaem et al., 2020). In addition, high-performance liquid chromatography (HPLC) analysis was used to determine the glucose and xylose concentrations in the supernatant (Khomlaem et al., 2023).

Separate hydrolysis and fermentation

S. cerevisiae TISTR 5606 samples were cultured in 250 mL Erlenmeyer flasks containing 10 mL of 10% (volume per volume) inoculum and 90 mL of the YPD liquid medium at pH 5.0. OPEFB hydrolysate was added to the YPD liquid medium and the culture was then incubated at 30°C with continuous shaking at a rate of 150 rpm. Based on the much prior expertise with this yeast and the available data, it was determined that the fermentation temperature and pH utilized were optimal (Azhar et al., 2017). Therefore, numerous Erlenmeyer flasks for fermentation were started. Three flasks were collected at various times, from which the glucose, xylose and bioethanol concentrations in the broth culture were measured.

Simultaneous saccharification and fermentation

Each sample (10 g) of the treated OPEFB was added to 100 mL of a buffer solution containing 50 mM sodium citrate buffer at pH 5.0 in a 250 mL Erlenmeyer flask. The mixture was sterilized by autoclaving for 15 min at 121°C and cooled to 37°C. The optimum enzyme concentration was added with 10% inoculum of *S. cerevisiae* TISTR 5606 and shaken at 150 rpm for 72 hr. The incubation temperature and pH were 37°C and pH 5.0, respectively. Several Erlenmeyer flasks were used to conduct parallel runs, with samples being collected at different times to quantify glucose and bioethanol concentrations.

Delayed simultaneous saccharification and fermentation

Each sample (10 g) of the treated OPEFB was suspended in 100 mL of the 50 mM sodium citrate buffer at pH 5.0 in a 250 mL Erlenmeyer flasks and sterilized in an autoclave for 15 min at 121°C. The slurry was heated to 50°C and the optimal enzyme concentration was introduced at the beginning of the process and sustained for 12 hr. Subsequently, the temperature was reduced to 37°C and 10% inoculum of *S. cerevisiae* TISTR 5606 was added, after which the reaction proceeded for another 60 hr amounting to an entire processing time of 72 hr. The glucose and bioethanol concentrations in samples were analyzed in triplicate in all three strategies.

Glucose, xylose and reducing sugar analysis

A Rezex RPM monosaccharide column (00H-0135-K0; 300×7.8 mm; USA) was used for HPLC (TFS-SY-0390 model; Thermo Finnigan; USA) with a refractive index detector and a mobile phase of ultrapure water at a flow rate of 0.6 mL/min to measure glucose and xylose according to Khomlaem et al. (2021). The reducing sugars were measured using DNS method, according to Miller (1959). Pure glucose at concentrations in the range 20–100 g/L and pure xylose at 5–20 g/L were used as standards.

Bioethanol analysis

The bioethanol concentration was based on HPLC analysis using a Bio-Rad column (Aminex HPX-87P; 300×7.8 mm; USA) and a refractive index detector with a mobile phase of 5 mM sulfuric acid at a constant flow rate of 0.6 mL/min. The column temperature was 50°C, with an injection volume of 10 µL (Bauer and Ibáñez, 2015). Pure ethanol at 5–30 g/L concentrations was used as a standard.

Statistical analysis

Analysis of variance was applied to analyze the data using the Statgraphics® Plus software (version 5.1; Manugistics Inc.; Rockville, MD, USA). Mean comparison was performed using *Fisher's test*. Differences between means were considered significant when the confidence interval was smaller than 5% ($p < 0.05$).

Results and Discussion

Composition of oil palm empty fruit bunch

The composition of OPEFB was significantly different before and after pretreatment (Table 1). The initial OPEFB contained 43.3 ± 0.89% cellulose, 20.3 ± 0.67% hemicellulose and 13.8 ± 0.65% lignin. The high cellulose content indicated that OPEFB was a suitable material for hydrolysis. However, removing the lignin component requires a pretreatment process. Several studies (Khalil et al., 2011; Thanapimmetha et al., 2012; Loh and Choo, 2013) have reported that an alkaline pretreatment can effectively reduce the lignin and hemicellulose contents, due to the NaOH in the alkaline process causing material swelling and increasing the internal surface area, while decreasing crystallinity and the degree of polymerization.

Furthermore, NaOH damages the structural links between lignin and carbohydrates and disrupts the lignin structure (Alvira et al., 2010). As seen in Table 1, after pretreatment, there was a 24% reduction in the OPEFB lignin content and an increase in the dry weight percentage of cellulose of 1.67 times compared to the untreated OPEFB. Small amounts of remaining lignin led to a low amount of cellulose-degrading enzymes being needed in the saccharification process (Mussatto et al., 2008; Alvira et al., 2010). The pretreatment results showed that OPEFB could be a viable, renewable carbon source that was suitable as a substrate for hydrolysis and fermentation.

Table 1 Untreated and pretreated oil palm empty fruit bunch (OPEFB) composition

Component	Dry composition (%, weight per weight)	
	Untreated OPEFB	Pretreated OPEFB with 10% NaOH
Cellulose	43.3 ± 0.89[b]	72.1 ± 0.70[a]
Hemicellulose	20.3 ± 0.67[a]	10.1 ± 0.64[b]
Lignin	13.8 ± 0.65[a]	10.5 ± 0.32[b]
Ash	1.09 ± 0.06[a]	0.07 ± 0.01[b]
Other	21.51 ± 0.37[a]	7.23 ± 0.27[b]

Mean±SD ($n = 3$) in each row superscripted with different lowercase letters are significantly ($p < 0.05$) different.

Enzymatic hydrolysis

The Cellic® CTec2 enzymes contained hemicellulase, cellulase and β-glucosidase, which were necessary to hydrolyze the treated lignocellulosic biomass. Fig. 2 illustrates the reducing sugar and glucose concentrations during 96 hr of hydrolysis for the different enzyme loadings of 5 FPU/g, 10 FPU/g and 15 FPU/g substrate. The enzyme loading significantly influences the kinetics of the hydrolysis of pretreated lignocellulosic biomass (Thanapimmetha et al., 2019). There was a significant increase reducing sugar production when the the enzyme concentration was increased from 5 FPU/g to 15 FPU/g substrate. Modenbach (2013) reported similar results. However, no there was no significant difference in the release of reducing sugar and glucose between 10 FPU/g and 15 FPU/g substrate (Figs. 2A and 2B). Exceeding the optimum loading negatively impacted the economic feasibility of the process, as the enzyme could not be recovered and it accounted for nearly 50% of the overall cost involved in the production of lignocellulosic bioethanol (Karimi and Chisti, 2017). Therefore, the enzyme concentration of 10 FPU/g substrate was chosen as the optimum condition.

At the optimal enzyme loading of 10 FPU/g substrate, the reducing sugar concentration was 74.2 ± 2.96 g/L after 96 hr, which was 2.41-fold higher than reported by Khomlaem et al. (2023), though they used 10% w/v NaOH for their corn cob pretreatment and obtained 30.8 g/L of reducing sugar concentration with their optimal conditions. Using 10 FPU/g substrate produced 58.1 ± 0.61 g/L and 9.60 ± 0.71 g/L of glucose and xylose, respectively (Fig. 2). A high glucose concentration at hydrolysis was preferable because the current study used *S. cerevisiae* TISTR 5606 fermentation.

Furthermore, it was observed that after 12 hr of treatment with 10 FPU/g substrate of enzymes, the hydrolysate had a reducing sugar concentration of 20 g/L (Fig. 2A) and the glucose concentration approached 20 g/L (Fig. 2B). This glucose concentration is commonly found in a medium used to cultivate *S. cerevisiae* TISTR 5606. Therefore, adding yeast to the hydrolysate at 12 hr was deemed suitable to initiate the DSSF strategy.

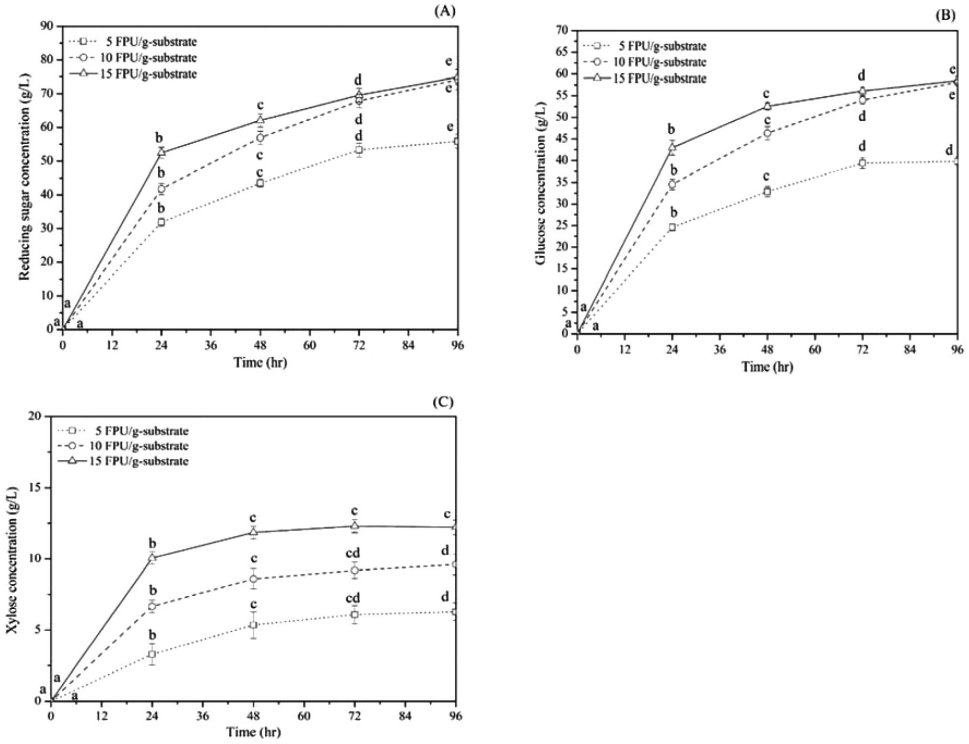

Fig. 2 Enzyme hydrolysis of oil palm empty fruit bunch pretreated with 10% NaOH at various enzyme loadings: (A) reducing sugar; (B) glucose; and (C) xylose, where data are shown as mean±SD (n = 3), different lowercase letters indicate significant ($p < 0.05$) differences among fermentation times, and FPU = filter paper units

Separate hydrolysis and fermentation

The fermentation pattern of the OPEFB hydrolysate from the previous section is illustrated in Fig. 3. All the glucose was rapidly used, resulting in nearly complete fermentation within 12 h, with the maximum bioethanol concentration being 24.0 ± 0.11 g/L ($p < 0.05$), as shown in Fig. 3.

Xylose was not consumed because it cannot be digested by *S. cerevisiae* (Vedernikovs et al., 2023). Overall, the bioethanol production in this study was 2.83-folds higher than that reported by Duangwang and Sangwichien (2015) of 8.49 g/L using treated OPEFB (NaOH 15%w/v), H_2SO_4 hydrolysis and fermentation with baker's yeast. Differences in the pretreatment and hydrolysis methods may have been responsible for the differences in bioethanol production.

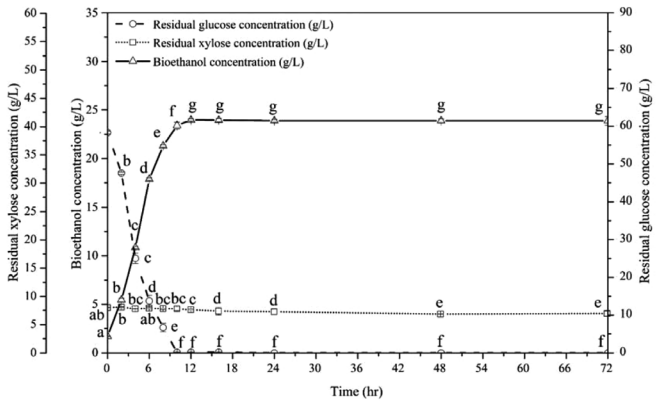

Fig. 3 Time courses of separate hydrolysis and fermentation (SHF) at 30°C and pH 5.0, where data are shown as mean±SD (n = 3) and different lowercase letters indicate significant ($p < 0.05$) differences among fermentation times.

Simultaneous saccharification and fermentation

The profiles of the SSF strategy are shown in Fig. 4. The enzyme and yeast were added together at the beginning and the incubation temperature remained at 37°C throughout the SSF process (Wang et al., 2013). As shown in Fig. 4, the substrate's enzymatic hydrolysis caused higher glucose consumption than the yeast growth rate. Therefore, early glucose consumption caused the glucose concentration to spike at 4 hr, as the growing cells rapidly took up glucose. After 8 hr, all the had been glucose consumed and the bioethanol increased to its maximum of 22.8 ± 0.30 g/L by the end of fermentation at 72 hr ($p < 0.05$), as shown in Fig. 4. The obtained bioethanol concentration was comparatively higher than that obtained by Christia et al. (2016), who reported a bioethanol production of 16.9 g/L using 8% NaOH pretreatment of OPEFB and an SSF strategy.

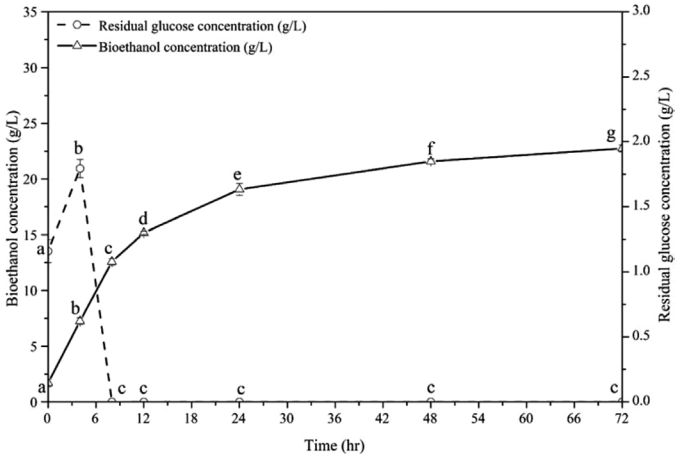

Fig. 4 Time courses of simultaneous saccharification and fermentation (SSF), where fermentation strategy involved adding enzyme and yeast together at beginning of process at 37°C and pH 5.0, data are shown as mean±SD (n = 3), and different lowercase letters indicate significant ($p < 0.05$) differences among fermentation times.

Delayed simultaneous saccharification and fermentation

The DSSF strategy aimed to partially hydrolyze the substrate using enzymes under saccharification-optimized conditions and then to lower it to the best temperature for fermentation once a specific concentration of sugars had been freed. Then, the saccharification fermentation process can be maintained simultaneously while being inoculated with the fermenting microorganism at a set temperature. In the DSSF strategy, the glucose concentration increased quickly due to enzymatic hydrolysis of the substrate during the first 12 hr. The glucose concentration reached 20.9 ± 0.50 g/L by 12 hr (Fig. 5). After the temperature had been lowered to 37°C, the yeast was added and the glucose continued to be consumed. Most of the glucose was consumed entirely by 48 hr. The final bioethanol production at 72 hr was 26.1 ± 0.18 g/L ($p < 0.05$), as presented in Fig. 5. The bioethanol production by DSSF in the current study was 1.31-folds higher than that reported by Fatriasari et al. (2018), who used the same DSSF strategy. They obtained a lower bioethanol yield of 19.9 g/L due to their shorter hydrolysis time (4 hr).

Comparison among SHF, SSF and DSSF strategies

Two optimum temperatures (50°C and 30°C) were used in the SHF strategy (Azhar et al., 2017). However, the SSF and DSSF strategies used 37°C as a compromise between saccharification and yeast fermentation, according to Thanapimmetha et al. (2019). Table 2 summarizes the time required for the three fermentation strategies to produce the same 22.8 ± 0.30 g/L maximum level of bioethanol production from the SSF strategy, which was the lowest bioethanol production among the three. SHF was the time-consuming fermentation strategy, needing 106 hr (Table 2). However, compared to the SHF strategy, SSF and DSSF could reduce the overall time by 31.1 and 38.7%, respectively, (Table 2), which was satisfactory.

The results of the three fermentation strategies for bioethanol production are summarized in Table 3. The DSSF was significantly superior to the other two fermentation strategies ($p < 0.05$), achieving approximately 9% greater bioethanol concentration than the SHF strategy. In addition, it had the highest bioethanol productivity of 0.36 g/L/hr (Table 3).

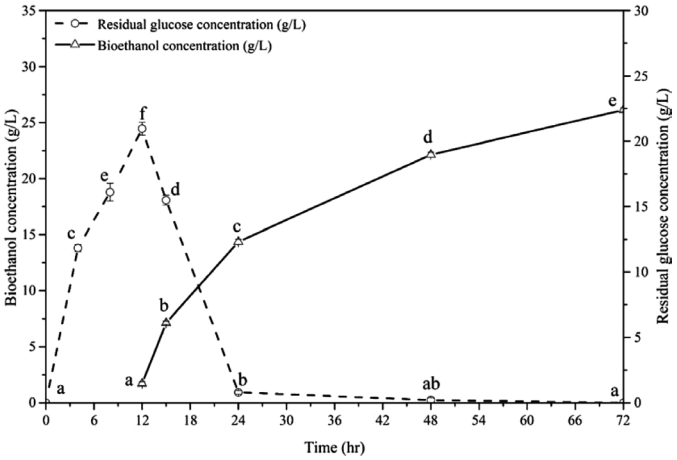

Fig. 5 Time courses of delayed simultaneous saccharification and fermentation (DSSF), where fermentation strategy involved hydrolysis for 12 hr at 50°C and combined hydrolysis-fermentation at 37°C, data are shown as mean±SD (n = 3), and different lowercase letters indicate significant ($p < 0.05$) differences among fermentation times.

Table 2 Time to obtain 22.8 ± 0.30 g/L of bioethanol using each strategy

Fermentation strategy	Pretreatment time (hr)	Hydrolysis time (hr)	Fermentation time (hr)	Hydrolysis and fermentation time (hr)	Total time (hr)	Time reduction relative to SHF (%)
SHF	1.00	96.0	9.00	-	106	-
SSF	1.00	-	-	72.0	73.0	31.1
DSSF	1.00	12.0	-	52.0	65.0	38.7

SHF = separate hydrolysis and fermentation; SSF = simultaneous saccharification and fermentation; DSSF = delayed simultaneous saccharification and fermentation.

Table 3 Ethanol production from three different fermentation processes

Fermentation strategy	Bioethanol production (g/L)	Yield (% of theoretical)	Yield coefficient (g bioethanol/g cellulose)	Hydrolysis and fermentation time (hr)	Bioethanol productivity (g/L/hr)
SHF	24.0 ± 0.11[b]	72.3 ± 0.34[b]	0.33 ± 0.002[b]	108 (96 + 12)	0.22 ± 0.001[c]
SSF	22.8 ± 0.30[c]	68.6 ± 0.92[c]	0.32 ± 0.004[c]	72	0.32 ± 0.004[b]
DSSF	26.1 ± 0.18[a]	78.8 ± 0.55[a]	0.36 ± 0.003[a]	72 (12 + 60)	0.36 ± 0.003[a]

SHF = separate hydrolysis and fermentation; SSF = simultaneous saccharification and fermentation; DSSF = delayed simultaneous saccharification and fermentation. Mean±SD (n = 3) in columns superscripted with different lowercase letters indicating significant ($p < 0.05$) differences.

SHF was the second-best fermentation strategy based on bioethanol production because the substrate was hydrolyzed separately. On the other hand, the SSF process performed the worst since the constant temperature of **37**°C was much lower than optimal for hydrolysis.

Conclusion

This study produced bioethanol from oil palm empty fruit bunch (OPEFB), a waste oil palm substrate. The treated OPEFB contained 72.1 ± 0.70% cellulose, 10.1 ± 0.64% hemicellulose and 13.8 ± 0.65%. Of the three fermentation strategies compared SHF performed enzymatic hydrolysis and fermentation sequentially; however, this was time-consuming. SSF combined enzymatic hydrolysis with fermentation in a single step, with a resultant time reduction. The DSSF strategy (two-temperature steps in one operation) improved the bioethanol production from the SSF strategy since the optimum temperature of enzymatic hydrolysis is typically higher than the fermentation temperature. The significantly greatest bioethanol production was by the DSSF strategy (26.1 ± 0.18 g/L) with corresponding productivity of 0.36 g/L/hr. In addition, its bioethanol concentration was 1.09 times higher than for SHF. Thus, DSSF is a promising fermentation strategy for bioethanol production from OPEFB with the minimum overall industrial bioethanol production process time of the three methods investigated.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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