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Utilization of pretreated oil palm empty fruit bunches and their hydrolysate for ethanol production by Indonesian ethanologenic yeast

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Abstract. *Anita SH, Oktaviani M, Hermiati E. 2023. Utilization of pretreated oil palm empty fruit bunches and their hydrolysate for ethanol production by Indonesian ethanologenic yeast. Biodiversitas 24: 5243-5252.* Oil Palm Empty Fruit Bunches (OPEFB) represent a polysaccharide-rich raw material with promising potential for ethanol production. This study aimed to investigate the ethanologenic yeasts, specifically *Saccharomyces cerevisiae* InaCC Y93 and *Kluyveromyces marxianus* InaCC Y119, affect bioethanol production in three different systems: Separate Hydrolysis and Fermentation (SHF), Simultaneous Saccharification and Fermentation (SSF), and Prehydrolysis-Simultaneous Saccharification and Fermentation (PSSF). This work is distinguished by the use of indigenous Indonesian yeast strains, including a thermotolerant strain. In the pretreatment process, 1.13% oxalic acid was added to OPEFB and subjected to microwave treatment at 190°C for 3.01 min. Subsequently, cellulase enzymes (40 FPU/g) and a 10% (w/v) yeast inoculum were introduced into 5.27 g dry weight of pretreated OPEFB pulp. The OPEFB acid hydrolysate was also subjected to fermentation. Ethanol content was monitored at 24 h intervals for 72 h. The PSSF system employs *K. marxianus* InaCC Y119 at 48 h exhibited the highest ethanol concentration, yielding 0.290 g/g, equivalent to approximately 51.20% of the theoretical yield. Additionally, *K. marxianus* InaCC Y119 demonstrated its capability to ferment the OPEFB acid hydrolysate into ethanol. These findings underscore the considerable potential of *K. marxianus* for applications in fermenting both hexose and pentose sugars to produce ethanol within higher-temperature systems.

Keywords: Bioethanol, *Kluyveromyces marxianus*, *Pachysolen tannophilus*, *Saccharomyces cerevisiae*

Abbreviations: GHG: Greenhouse Gas; OPEFB: Oil Palm Empty Fruit Bunches; PSSF: Pre-Hydrolysis-Simultaneous Saccharification and Fermentation; SHF: Separate Hydrolysis and Fermentation; SSF: Simultaneous Saccharification and Fermentation; SSCF: Simultaneous Saccharification and Co-Fermentation; CBP: Consolidated Bioprocessing; MEMR: Minister of Energy and Mineral Resources; PDA: Potato Dextrose Agar; YPD: Yeast Peptone Dextrose; OD: Optical Density; DNS: Dinitro Salicylic Acid; HMF: Hydroxymethyl Furfural

INTRODUCTION

The massive increase in population, from 2.7 billion in 1955 to 8.0 billion in 2022, has placed considerable demands on resources and their consumption (United Nations 2022). Since the mid-2000s, Indonesia has become a net oil importer due to rising domestic energy demand. The Indonesian government has taken significant steps toward energy diversification, which include the substitution of petroleum with biofuels. Presidential Regulation No. 5/2006 sets a target of 5% biofuel utilization by 2025. Following the issuance of this regulation, mandatory regulations followed. The most recent of these, Minister of Energy and Mineral Resources (MEMR) Rule No. 12/2015, established new objectives for bioethanol to account for 20% of the total gasoline demand (Adiatma and Prasojo 2021). Bioethanol has great potential as a renewable energy alternative to petroleum in the transportation sector and is ideal for use as a blended fuel in gasoline engines. Bioethanol produces little to no net carbon dioxide, helping to reduce Greenhouse Gas (GHG) emissions in the environment. Ethanol's partial substitution in gasoline reduced GHG emissions from transportation by 43.5 million metric tons in 2016, which is equivalent to taking 9.3 million cars off the road for a single year (Rocha-Meneses et al. 2017; Robak and Balcerek 2018; Trisna et al. 2022).

The production of lignocellulosic bioethanol is still in high demand due to its low cost and lack of competition with the food supply (Robak and Balcerek 2018; Lamichhane et al. 2021). Lignocellulosic biomasses, such as corn cob, rice husk, cassava peels, sugar cane bagasse, and yam peels (Awoyale and Lokhat 2021), along with Oil Palm Empty Fruit Bunches (OPEFB) (Sukhang et al. 2020; Irwan and Salim 2021; Suhartini et al. 2022), are all being considered as prospective and sustainable sources for bioethanol production. OPEFB is a potential substrate for bioethanol fermentation due to its rich polysaccharides and abundant availability in Indonesia. Indonesian palm oil companies produced approximately 40 to 50 million tons of OPEFB in 2020 (Maryana et al. 2021).

Converting lignocellulosic biomass into ethanol is

challenging, requiring feedstock pretreatment before fermentation and the development of ethanol microorganisms capable of fermenting both hexose and pentose sugars (Robak and Balcerek 2018). The pretreated lignocellulosic biomass can be converted into ethanol using various process configurations, including Separate Hydrolysis and Fermentation (SHF), Simultaneous Saccharification and Fermentation (SSF), Pre-Hydrolysis-Simultaneous Saccharification and Fermentation (PSSF), Simultaneous Saccharification and Co-Fermentation (SSCF), and Consolidated Bioprocessing (CBP) (Silva et al. 2015; Su et al. 2020; Broda et al. 2022). While SSCF and CBP require further research in biorefineries and biotechnology centers, SSF is the most cost-effective and recommended alternative for ethanol production (Robak and Balcerek 2018; Broda et al. 2022). The SSF technique is recognized for reducing contamination risk and saving costs by using a single reactor for both hydrolysis and fermentation (Silva et al. 2015).

The SSF method has been found to yield more ethanol than the SHF process. However, SSF is limited by the typically inadequate conditions for hydrolysis and fermentation (Broda et al. 2022). Enzymes for cellulose hydrolysis have an optimum temperature range of 45-50°C, whereas the optimum temperature for fermentation falls in the range of 28-37°C (Tran et al. 2019; Lamichhane et al. 2021). PSSF was developed to address the issue of hydrolysis during the SSF process, which often occurs at temperatures lower than the optimum (Su et al. 2020).

Saccharomyces cerevisiae is a yeast commonly used for ethanol production, either in free cells (Kim et al. 2014; Sandoval-Nuñez et al. 2017) or immobilized in the matrix (Kumoro et al. 2021). The processes of ethanol fermentation by *S. cerevisiae* are favorable at temperatures ranging from 30 to 35°C, which is incompatible with the SSF process (Nachaiwieng et al. 2015). Therefore, thermotolerant yeasts are recommended for use in SSF or PSSF procedures. *Kluyveromyces marxianus* is a thermotolerant ethanol-fermenting yeast. *Kluyveromyces marxianus* can produce ethanol at temperatures up to 40 to 50°C. However, *K. marxianus* is sensitive to high levels of ethanol. Unlike *S. cerevisiae*, which can only ferment hexose sugar, *K. marxianus* is capable of metabolizing various substrates, including hexose sugars (glucose, galactose, lactose, and mannose) and pentose sugars (arabinose and xylose) (Bilal et al. 2022). Similar to *K. marxianus*, *Pachysolen tannophilus* can ferment xylose to ethanol. However, it cannot tolerate high temperatures and high ethanol concentrations (Baig and Smita 2018).

In this study, we investigated the ethanol production process from pretreated OPEFB using three alternative fermentation systems: SHF, SSF, and PSSF. The aim was to identify a more effective and efficient ethanol production process. The use of indigenous Indonesian yeast, including a thermotolerant strain, is another feature that sets this work apart. The goal of this study was to investigate the ethanol production from pretreated OPEFB pulp using the SHF, SSF, and PSSF methods with the yeasts *Kluyveromyces marxianus* InaCC Y119 and *Saccharomyces cerevisiae* InaCC Y93. After the pretreatment process, the OPEFB acid hydrolysate was fermented with *Pachysolen tannophilus* InaCC Y114 and *Kluyveromyces marxianus* InaCC Y119. This method allows us to find possible yeasts that can be co-cultured to produce ethanol from both hexose and pentose sugars. This way, we can improve the process of making ethanol by using the right systems.

MATERIALS AND METHODS

Raw materials, microorganisms, and chemicals

OPEFB was obtained from Sukabumi, West Java, Indonesia. *Saccharomyces cerevisiae* InaCC Y93, *Kluyveromyces marxianus* InaCC Y119, and *Pachysolen tannophilus* InaCC Y114 were obtained from the Indonesia Culture Collection (InaCC), National Research and Innovation Agency (BRIN), Bogor, Indonesia. The enzyme used for the hydrolysis process was meicellase from *Trichoderma viride*, supplied by Meiji Seika Co., Ltd. Tokyo, Japan. Yeast extract, Potato Dextrose Agar (PDA), glucose, xylose, peptone, $MgSO_4$, $CaOH_2$, and oxalic acid were purchased from Merck (Darmstadt, Germany).

Preparation of raw material

Before use, OPEFB was air-dried, ground with a hammer mill, and sieved through a 40-60 mesh sieve. The moisture content of the raw material was 5.44 ± 0.09%. The prepared raw material was then kept at room temperature in a sealed container to retain its moisture content. The composition of raw materials has been previously reported by Solihat et al. (2017), which contains 18.29% lignin, 42.6% cellulose, and 24.46% hemicellulose.

Pretreatment process

Pretreatment using oxalic acid was conducted according to the method described earlier by Anita et al. (2020). Three grams of OPEFB (40-60 mesh) were inserted into a teflon vessel, and then 30 mL of 1.13% oxalic acid was added to achieve a solid-liquid ratio (solid loading) of 1:10. The pretreatment process was performed using microwaves (Milestone START D, Milestone Inc., Bergamo, Italy) at 190°C for 3.01 min. After completing the pretreatment process, the pretreated OPEFB pulp was separated from the hydrolysate by vacuum filtration (GAST DOA-P504-BN, Cole-Parmer, Vernon Hills, IL, USA). It was then washed with distilled water until the wash water reached a pH of 6.0. The pretreated OPEFB pulp was subsequently used for the enzymatic hydrolysis process, while the OPEFB acid hydrolysate was designated for the fermentation process. The pulp recovery of pretreated OPEFB was 50.39%, and it contained 61.12% cellulose.

Preparation of culture starter for SHF, PSSF, and SSF

A loop full of each *Saccharomyces cerevisiae* InaCC Y93 and *Kluyveromyces marxianus* InaCC Y119 was added to 100 mL of 5% YPD medium in a 500 mL flask. The culture starter was then incubated in a shaking incubator at 120 rpm and 30°C for 16-20 h (Bio-Shaker BR-300, Japan). Before use, yeast cells from the culture starter were separated using a centrifuge at 10,000 rpm at

4°C for 10 min. The yeast cells were then re-suspended in 1/10th sterile distilled water of the initial medium volume. The OD inoculum was measured using a spectrophotometer (Hitachi U-2001, Hitachi Instruments Inc., Tokyo, Japan) at λ = 600 nm. The OD inoculum was maintained below 0.800, corresponding to the log/exponential phase. The suspension was an inoculum for the fermentation process.

Separate hydrolysis and fermentation

An amount of 5.27 g dry weight of pretreated OPEFB pulp was weighed in a 250-mL flask. Sodium azide 2%, sodium citrate buffer (pH 4.8) 0.05 M, and 40 FPU/g of enzyme cellulase were added into the flask until it reached 50 g of solution. The same preparation was also performed for the enzyme control (without substrate). The flask was sealed and incubated in a shaking incubator at 150 rpm, 50°C for 72 h. Fermentation was carried out after the completion of the hydrolysis process. The slurry obtained after hydrolysis was sterilized by autoclaving at 121°C for 15 min. Subsequently, a 10% (v/v) inoculum of *S. cerevisiae* was inoculated to the sterilized slurry. Each flask was then covered by bubble traps and incubated in a shaking incubator at 120 rpm, 30°C for 72 h.

Simultaneous saccharification and fermentation

A total of 5.27 g dry weight of pretreated OPEFB pulp was weighed in a 250-mL flask. The pretreated pulp was first sterilized by heating under pressure at 121°C for 15 min. After cooling, the flask containing the sterile pretreated OPEFB pulp was weighed again to determine the loss of water due to pressure heating. The fermentation medium (5 mL) consisting of 100 g/L yeast extract, 200 g/L peptone, 0.1 M sodium citrate buffer (2.5 mL), and a 10% (v/v) inoculum of yeast *K. marxianus*, along with cellulase (meicellase) enzyme (40 FPU/g), were added to the flask containing the pretreated pulp. Sterile distilled water was added to the flask until the total weight of the mixture reached 50 g. Except for enzymes, the fermentation media and buffer solutions used were sterilized at 121°C for 15 min. The same preparation was carried out for enzyme control (without substrate). The flask was then equipped with a bubble trap to capture any gases produced. The SSF process was carried out in a shaking incubator (Bio-Shaker BR-300, Japan) at 150 rpm and 38°C for 72 h. Sample filtrate was taken at 1 mL intervals every 24 h for 72 h to analyze the sugar and ethanol concentrations.

Prehydrolysis simultaneous saccharification and fermentation

An amount of 5.27 g dry weight of pretreated OPEFB pulp was weighed in a 250 mL flask and sterilized. Pre-hydrolysis was done by adding 0.05 M sodium citrate buffer (pH 4.8) and cellulase enzyme 40 FPU/g into the pretreated sample, which was then incubated in a shaking incubator at 150 rpm, 50°C for 3 h. After pre-hydrolysis was completed, a fermentation medium (5 mL) consisting of 100 g/L yeast extract, 200 g/L peptones, and 10% (v/v) yeast inoculum of *S. cerevisiae* and *K. marxianus* each was added to the flask. Sterile distilled water was added to the flask to achieve a total weight of 50 g. The fermentation medium was sterilized at 121°C for 15 min. The flask was sealed with bubble traps and incubated in a shaking incubator at 150 rpm, 38°C for 72 h.

Fermentation of OPEFB acid hydrolysate

Pachysolen tannophilus InaCC Y114 was cultured in a medium containing 1 g/L $MgSO_4$, 2 g/L KH_2PO_4, 3 g/L $(NH_4)_2SO_4$, 3.6 g/L peptone, 4 g/L yeast extract, and 25 g/L xylose. The inoculum was prepared by inoculating a loop full of yeast into 100 mL of medium in a 500 mL flask and incubating it in a shaking incubator at 150 rpm, 35°C for 16-20 h. $Ca(OH)_2$ was subjected to OPEFB acid hydrolysate pretreatment until pH 7.0 was achieved and then re-adjusted to pH 4.5. Before the sterilization process, yeast extract at a concentration of 1 g/L was also added to the hydrolysate. Five milliliters of *P. tannophilus* were inoculated into 95 mL of hydrolysate in a 300 mL flask. The flask was equipped with bubble traps and incubated in a shaking incubator at 150 rpm, 35°C for 72 h. The same process was performed for *K. marxianus*. Samples were collected every 24 h for 72 h to analyze the sugar and ethanol content.

Analytical methods

Samples obtained after fermentation were centrifuged at 10.000 rpm for 10 min. The supernatant was used to measure the reducing sugar and ethanol concentrations. The reducing sugar concentration was determined using the Dinitro Salicylic Acid (DNS) method (Miller 1959) and measured using a UV-Vis spectrophotometer at 540 nm. Ethanol concentration was measured using Gas-Chromatography (GC). The ethanol concentration was determined using the following formula (Fatriasari et al. 2020):

Ethanol concentration (g/L) = Ethanol content (v/v) (%) x 10 x 0.789

The ethanol yield and percentage of theoretical ethanol for the SHF, PSSF, and SSF process were calculated by the following formula:

Ethanol yield (g/g) = g of ethanol /g cellulose in working volume ((Wix100)/Pr) x Cf)

Where: Wi: initial dry weight of biomass (g), Pr: pulp recovery, and Cf: cellulose fraction of dry biomass (g).

Percentage of theoretical ethanol (%) =

$$\left(\frac{\text{Ethanol yield (g/g)}}{\text{Theoretical of ethanol yield (cellulose in substrate x 1.11 * x 0.51 **) (g/g)}}\right) x100$$

Where: *1.11 is a correction factor to compensate for the addition of water molecules after the breakage of cellulose into glucose monomers, **0.51 is a correction factor to represent the maximum possible ethanol yield from glucose based on the stoichiometric ratio between ethanol and glucose in glucose fermentation

The sugar consumption was calculated as the ratio between the amount of sugar used and the amount of initial sugar multiplied by 100 for the fermentation of OPEFB acid hydrolysate. The ethanol yield (g/g) was calculated as grams of ethanol produced per gram of consumed sugar, and the percentage of theoretical ethanol (%) was estimated by the ratio of the ethanol yield to the theoretical value of the ethanol yield (0.51 g/g).

GC analysis

Ethanol content analysis was done using Gas Chromatography (GC-2010 Plus Shimadzu, Kyoto, Japan) with an RTX-Wax column. The column specifications were: length of column 30 m, inner diameter 0.25 mm, and film thickness 0.25 µm. The initial temperature of the column was 35°C. The injector and detector temperatures are respectively maintained at 200°C and 210°C. The sample injected was 1 µL with a total flow of 83.5 mL/min and a split ratio of 40.

Statistical analysis

The experiments were performed in triplicate. Afterward, the data were subjected to statistical analysis using analysis of variance (ANOVA) to determine their significance, and the means were compared using Tukey's test at the 5% level.

RESULTS AND DISCUSSION

OPEFB is a potential source of sugar and a suitable raw material for ethanol production. However, extracting simple sugars from lignocellulosic biomass presents significant challenges. The ethanol production process from lignocellulosic biomass, which is primarily composed of cellulose, hemicellulose, and lignin, involves three key steps: pretreatment, hydrolysis (also known as saccharification) of cellulose and/or hemicelluloses to yield simple sugars, and fermentation of these sugars to produce ethanol (Anita et al. 2020; Sukhang et al. 2020). In this study, microwave-oxalic acid (MOA) pretreatment was conducted to prepare OPEFB biomass with a high cellulose content. Anita et al. (2020) reported that MOA pretreatment caused disruptions in the OPEFB structure and dissolved the hemicellulose, facilitating the penetration of cellulase enzymes into the biomass. This enhanced the saccharification of cellulose into sugars.

In order to evaluate the fermentability of the pretreated OPEFB pulp, three different fermentation systems were employed: SHF, SSF, and PSSF. These systems utilized the microorganisms *S. cerevisiae* and *K. marxianus.* A detailed description of the fermentation outcomes for these three systems is presented below.

Separate hydrolysis and fermentation

The SHF method is divided into two stages: hydrolysis and fermentation. Enzymatic hydrolysis, also known as saccharification, is conducted to obtain simple sugars from cellulose. The glucose present in the hydrolyzed substance was subsequently subjected to fermentation, resulting in the production of ethanol (Dahnum et al. 2015). Enzymatic hydrolysis of pretreated OPEFB pulp after 72 h yielded a reducing sugar content of 59.92 g/L. Subsequently, these initial sugars were fermented using *Saccharomyces cerevisiae*. After 72 h of enzymatic hydrolysis, the process continued with fermentation, extending the total duration to 144 h. As the fermentation duration increased, the concentration of sugar dropped while the concentration of ethanol simultaneously increased (Figure 1A). Based on the result obtained, it was found that the maximum ethanol content reached 27.10 g/L after 72 h of fermentation, and the ethanol yield from pretreated OPEFB pulp in the SHF process was 0.212 g/g, approximately 37.41% of the theoretical yield (Figure 1B).

The results of this study are identical to those of Sudiyani et al. (2020). In the SHF study, the glucose concentration at the beginning of the fermentation phase was relatively high at 138 g/L, but after 72 h of fermentation, no glucose was present when the ethanol concentration reached its peak. It implies that *S. cerevisiae* consumes sugar and converts it to ethanol. Sudiyani et al. (2020) also reported a higher ethanol yield of 69.84 g/L, which was obtained from enzymatically hydrolyzed OPEFB during 72 h of fermentation in the SHF process. Additionally, a study by Siregar et al. (2019) on ethanol production from sulfuric acid-hydrolyzed OPEFB using *S. cerevisiae* showed that the highest ethanol concentration of 41.41 g/L was achieved after 96 h of fermentation. Several investigations have reported that *S. cerevisiae* produces varying amounts of ethanol from OPEFB. This variability can be influenced by numerous fermentation factors, including temperature, inoculum loading, agitation, pH, substrate loading, fermentation length, pretreatment, and the hydrolysis process (Sukhang et al. 2020; Faustine et al. 2021).

Fermentation in the SHF process exclusively employed *S. cerevisiae* and occurred at 30°C, in accordance with the mesophilic growth temperature range of *S. cerevisiae*, which is between 30°C and 40°C (Bhadana and Chauhan 2016). In their study, Boonchuay et al. (2021) documented that the thermotolerant strain *S. cerevisiae* TC-5, when isolated, had the ability to survive and produce bioethanol at a temperature of 40°C. Moreover, this strain shows a reduced need for additional nutrients and minerals for its growth and bioethanol production. However, numerous *S. cerevisiae* strains have shown that the optimal temperature for growth and ethanol production is between 30°C and 35°C (Durbha et al. 2014; Pornpukdeewattana et al. 2014; Sudiyani et al. 2020).

In this study, our focus was solely on the hydrolysis and fermentation systems, whether used separately, simultaneously, or preceded by the hydrolysis process. Several influencing factors, such as substrate loading, enzyme loading, inoculum size, duration of fermentation, as well as pretreatment and hydrolysis processes, were kept consistent to minimize their effect.

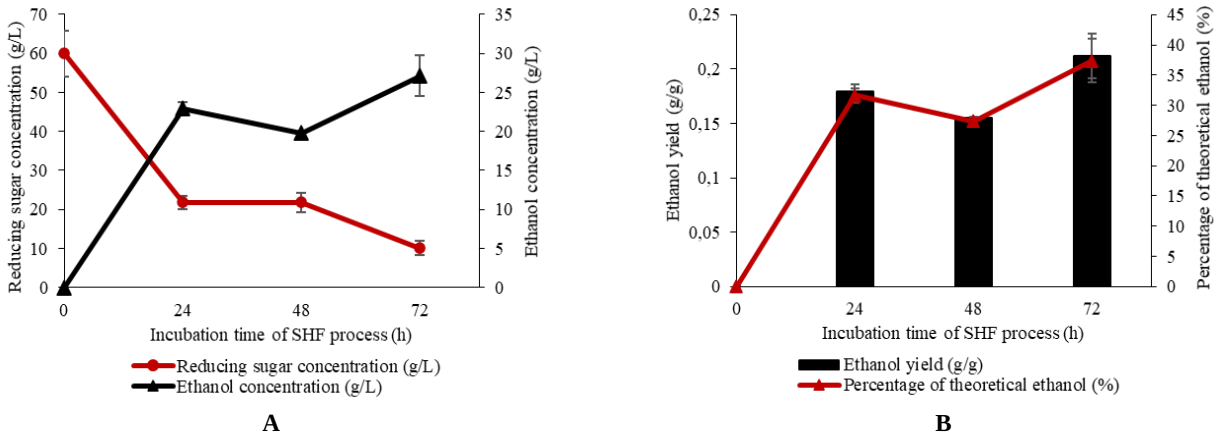

Figure 1. Reducing sugar and ethanol concentration (A), ethanol yield and percentage of theoretical yield (B) during SHF

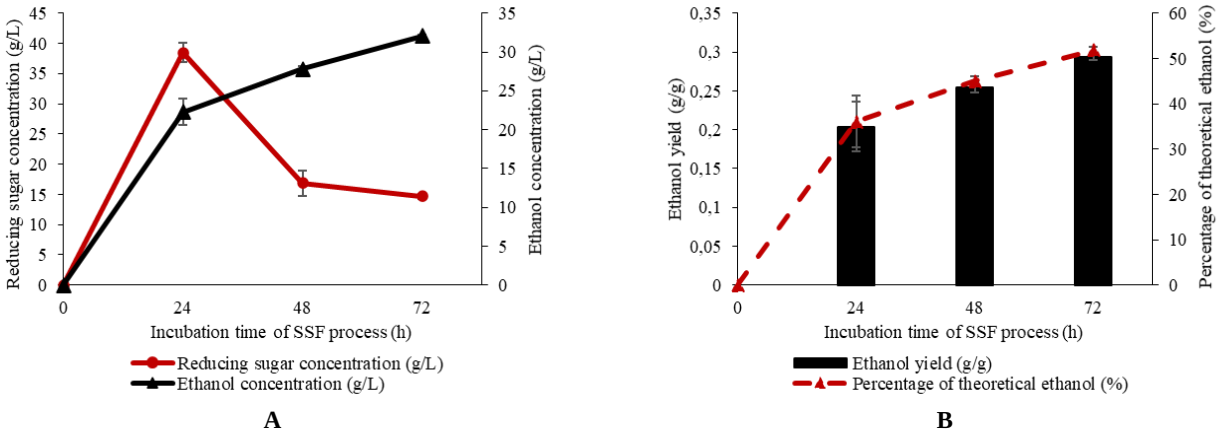

Figure 2. Reducing sugar and ethanol concentration (A), ethanol yield and percentage of theoretical yield (B) during SSF

SHF and SSF are the two most commonly used processes for producing bioethanol. SHF allows for optimal cellulase hydrolysis at 50ºC, which is higher than in SSF. As a result, enzyme activity increases, and the sugar yield may exceed 90% (Mejía-Barajas et al. 2018). Furthermore, fermentation in SHF takes place at 30ºC, which is the ideal temperature for *S. cerevisiae* to grow and convert sugar into ethanol. However, the SHF method uses two separate reactors for hydrolysis and fermentation, making it time- and cost-consuming. Therefore, SSF is offered as an alternative method that is more efficient in terms of both time and cost because the hydrolysis and fermentation processes occur simultaneously in a single reactor (Tomás-Pejó et al. 2010).

Simultaneous saccharification and fermentation

Pretreated OPEFB pulp was also subjected to fermentation via the SSF method, using the cellulase enzyme at 40 FPU and the non-traditional yeast strain *K. marxianus* at 38ºC. *Kluyveromyces marxianus* is employed in SSF due to its thermotolerance, ability to use various sugars as a carbon source, and its Generally Recognized as Safe (GRAS) status (Mejía-Barajas et al. 2018). The fermentation profile of *K. marxianus* using the SSF method was analyzed (Figure 2). The reducing sugar increased significantly during the first 24 h of fermentation due to the action of the cellulase enzyme (Akhtar et al. 2017). With the increased in the reducing sugar concentration, the ethanol concentration increased until it reached a concentration of 22.29 g/L in the first 24 h of fermentation. After 24 h, the reducing sugar decreased due to the increased sugar consumption by yeast (Fatriasari et al. 2018). However, the ethanol concentration kept increasing until 72 h of fermentation. The highest ethanol concentration obtained at 72 h was 32.06 g/L (Figure 2A). Similar to the ethanol concentration, the highest ethanol yield by *K. marxianus* was achieved at 72 h of fermentation, with a yield of 0.294 g/g. This yield was equivalent to approximately 51.28% of the theoretical yield value (Figure 2B). The ethanol concentration and yield may further increase with extended fermentation time beyond 72 h. However, for the purpose of comparison with other fermentation systems, the fermentation time was kept consistent.

Although the highest ethanol concentration in this study was obtained after 72 h of fermentation, the ethanol concentration at 48 h of fermentation was also quite high, reaching 27.82 g/L with a yield of 0.255 g/g. Our ethanol

value was identical to that reported by Sukhang et al. (2020), who studied ethanol production from OPEFB with *K. marxianus* TISTR5116. The ethanol concentration of 28.10 g/L with a yield of 0.281 g ethanol/g biomass was achieved after 48 h of fermentation. However, they used two pretreatment steps: acid pretreatment with 0.2 M H_2SO_4, followed by alkali pretreatment with 5% (w/v) NaOH, which probably resulted in higher costs and a longer production time. The ethanol concentration obtained in this research outperformed those of Gatdula et al. (2021), who got 6.30 g/L and 5.35 g/L of ethanol from the SSF of rice straw and banana pseudostem by *K. marxianus* for 48 h, respectively. Our results were also better than those reported by Hemansi et al. (2021), who found 20 g/L of ethanol during 24-72 h of SSF by *K. marxianus* using acid-alkali-pretreated cotton stalks.

According to the results shown above, the SSF method with *K. marxianus* appears to be more profitable than the SHF method with *S. cerevisiae*, particularly in terms of production time. However, the SSF method may still be limited by inadequate hydrolysis and fermentation conditions. These issues can be mitigated by incorporating a prehydrolysis phase into SSF and utilizing thermotolerant yeast (Murata et al. 2015; Liu et al. 2016). In this research, we employed a pre-hydrolysis method with both mesophilic yeast and thermotolerant yeast.

Prehydrolysis simultaneous saccharification and fermentation

The PSSF process involves a hydrolysis stage at the optimal enzyme temperature, followed by a mild-temperature stage in a single reactor, allowing SSF to overcome the limitations caused by differences in the optimal conditions for the microbe and enzyme (Tareen et al. 2021). In this study, pre-hydrolysis was conducted at 50°C for 3 h, followed by SSF at 38°C for 72 h. This PSSF technique employs two yeast strains: *S. cerevisiae* and the thermotolerant yeast *K. marxianus*.

The trends in reducing sugar, ethanol concentration, and ethanol yield during the PSSF process using *S. cerevisiae* and *K. marxianus* (Figure 3). Both fermentations exhibited similar trends in reducing sugar and ethanol concentrations. After 3 h of the pre-hydrolysis process, a reducing sugar concentration of 73.47 g/L was quickly attained (Figure 3A). The reducing sugar then decreased over the fermentation period due to yeast consumption. Both yeast strains achieved their highest ethanol concentration at 48 h of fermentation, resulting in a total duration of 51 h for a single operation process. However, PSSF uses *K. marxianus* yielded a higher ethanol concentration and ethanol yield than that achieved using *S. cerevisiae*. *K. marxianus* produced the highest ethanol concentrations and ethanol yield of 31.67 g/L (Figure 3A) and 0.290 g ethanol/g cellulose (Figure 3B), equivalent to 51.20% of the theoretical yield (Figure 3B). In contrast, PSSF uses *S. cerevisiae* produced an ethanol concentration of 26.96 g/L and an ethanol yield of 0.211 g/g cellulose, closely aligning with the theoretical value of 37.23% (Figure 3B).

K. marxianus InaCC Y119 exhibited a higher ethanol yield than *S. cerevisiae* InaCC Y93 at 38°C during 48 h of fermentation. Another study (Hashem et al. 2013) reported that *Kluyveromyces* sp. produces the highest ethanol content at 35°C after 48 h of fermentation. Murata et al. (2015) also noted that *K. marxianus* exhibits high fermentation activity at temperatures between 37°C and 45°C, while *S. cerevisiae* shows lower fermentation activity due to heat shock stress. *Kluyveromyces marxianus* species are tolerant of high temperatures and can survive in the range of 40°C to 52°C (Bilal et al. 2022). The advantages of thermotolerant yeasts for ethanol production include enhanced fermentation activity at elevated temperatures, which can reduce contamination in the bioreactor; lower cooling and distillation costs when fermentation is carried out at 40°C; and increased enzyme activity at higher temperatures, potentially reducing the need for additional enzyme supplementation (Murata et al. 2015). In this study, *K. marxianus* InaCC Y119 demonstrated superior ethanol production activity compared to *S. cerevisiae* InaCC Y93 at 38°C. Ethanol production at temperatures higher than 38°C using *K. marxianus* InaCC Y119 needs further investigation, as it could potentially be applied in an SSF system with a temperature closer to the cellulase's optimum temperature.

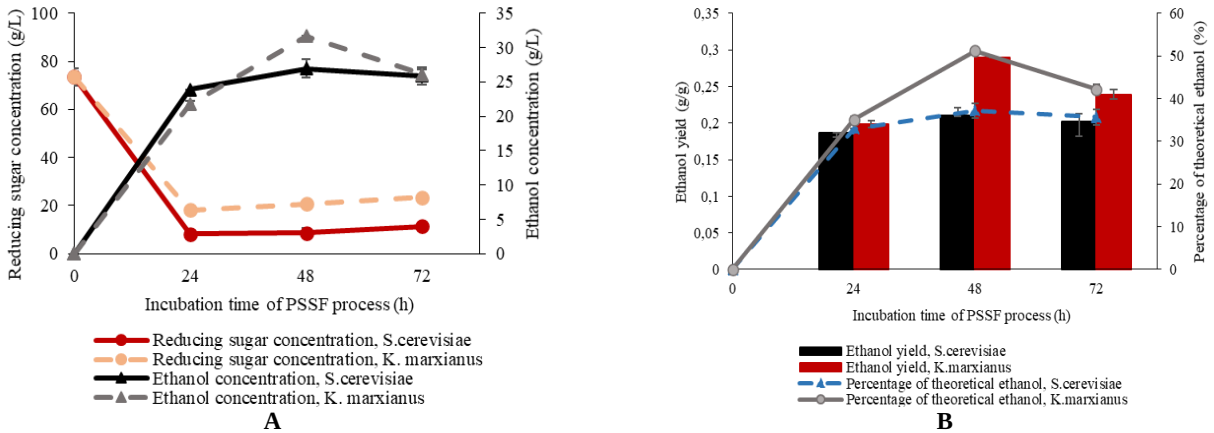

Figure 3. Reducing sugar and ethanol concentration (A), ethanol yield and percentage of theoretical yield (B) during PSSF

The ethanol yield generated by the SSF and PSSF systems utilizing *K. marxianus* was nearly identical, at approximately 0.29 g/g. However, the PSSF system requires less time than the SSF system. The findings align with those of Tareen et al. (2021), who observed that pre-hydrolysis decreases the slurry viscosity. In addition, the pre-hydrolysis process and the substrate supply period improved the efficiency of incorporation and the consistency between solid and enzyme loading. This led to more ethanol production and shorter fermentation times overall. The availability of simple sugars at the initial stage of fermentation might have accelerated the initiation of sugar transport across the yeast plasma membrane, leading to a shorter fermentation duration (Paschos et al. 2022). The primary simple sugars found following the OPEFB hydrolysis process are glucose and xylose. Previous research indicates that a significant amount of simple sugars, including xylose and glucose, is formed when OPEFB biomass is enzymatically hydrolyzed (Dahnum et al. 2015; Kim 2018; Sudiyani et al. 2020).

However, other studies have indicated that the ethanol yield was smaller or comparable in fermentation processes with or without pre-hydrolysis during the same period. Although the effect on ethanol output was minor, pre-hydrolysis accelerated liquefaction. Therefore, using pre-hydrolysis requires less stirring force than batch SSF without pre-hydrolysis (Gladis et al. 2015). PSSF is a variety of SSF processes that provide the short period of time necessary for lignocellulosic material to be partially hydrolyzed prior to fermentation. This method enables the use of higher temperatures during the initial enzymatic hydrolysis, which increases enzymatic activity. Another benefit of this process is the reduction in ethanol production time, which could increase the overall ethanol yield (Boonchuay et al. 2021).

In general, bioethanol production typically employs the *S. cerevisiae* strain, which is considered as a superior candidate. However, *K. marxianus* is an attractive candidate in the SSF system because it can ferment at higher temperatures, allowing for the utilization of optimal temperatures in the hydrolysis process for the cellulase (Sukhang et al. 2020). Table 1 compares the ethanol yields produced by several ethanologenic yeasts and the entire process of hydrolysis and fermentation. Based on the results obtained, there are several options for yeast types and operating systems that can impact ethanol yield and fermentation time. Regarding the overall process system, to obtain the highest ethanol concentration from pretreated OPEFB pulp, *K. marxianus* InaCC Y119 can be used as one of the alternative yeasts in the fermentation process. Nevertheless, the PSSF system is preferable to SSF for producing the highest amount of ethanol in a reduced amount of time.

During the process of chemical pretreatment, the production of five- and six-carbon sugars occurs alongside the formation of cellobiose and partly damaged cellulose. Achieving comprehensive and effective sugar utilization is a fundamental need for the economically viable manufacture of ethanol from biomass (Tesfaw and Assefa 2014). In this study, microwave pretreatment with oxalic acid on OPEFB fibers produced pretreated OPEFB pulp and acid hydrolysate. These two components can be used to produce ethanol through the utilization of yeast and different fermentation systems. The pretreated OPEFB pulp is used in the enzymatic hydrolysis process to make simple sugars. These sugars are then usually fermented with *S. cerevisiae*. Meanwhile, the OPEFB acid hydrolysate can be fermented to produce ethanol using specific yeast strains. However, OPEFB acid hydrolysate cannot be used directly as a fermentation substrate due to its high inhibitor concentration.

Chemical compounds in the hydrolysate pretreatment that inhibit the production of ethanol are furans, carboxylic acids, and phenolic compounds. Different approaches have been used to mitigate the inhibitory effects of these inhibitors in the production process. These strategies include strain selection, detoxification, adaptive evolution, co-culture or mixed culture, and optimization of process conditions (Tesfaw and Assefa 2014; Cunha et al. 2020). We used two methods in this study: first, we detoxified the OPEFB acid hydrolysate by adding $Ca(OH)_2$; this was then fermented; and second, we chose the strains by using *P. tannophilus* and *K. marxianus*. A more detailed explanation of the results of the OPEFB acid hydrolysate fermentation process is shown below.

Table 1. Comparison of ethanol yield by using different ethanologenic yeast and system processes of hydrolysis and fermentation

System process	Cellulase enzyme (FPU/g)	Hydrolysis temperature (°C)	Pre-hydrolysis time (h)	Ethanologenic yeast	Fermentation temperature (°C)	Optimum fermentation time (h)	Total time processes (h)	Ethanol yield (g/g)
SHF	40	50	72	*S. cerevisiae*	30	72	144	0.212[a]
SSF	40	38	—	*K. marxianus*	38	72	72	0.294[b]
PSSF	40	50	3	*S. cerevisiae*	38	48	51	0.211[a]
PSSF	40	50	3	*K. marxianus*	38	48	51	0.290[b]

Note: The mean value followed by the same letter is not significantly different according to the Tukey (HSD) test at the 0.05 significance level

Fermentation of OPEFB acid hydrolysate

Acid pretreatment typically generates acid hydrolysate containing a mixture of hexose (C_6) sugars such as glucose, mannose, and galactose, as well as pentose (C_5) sugars like xylose and arabinose (Ricciardi et al. 2022). Consequently, microorganisms capable of metabolizing both types of sugars and converting them into ethanol are necessary. Several yeast strains from the genera *Pachysolen*, *Pichia*, *Candida*, and *Kluyveromyces* have been reported to have the ability to convert both hexose and pentose sugars into ethanol (Musatto et al. 2012; Cuevas et al. 2020). *Pachysolen tannophilus* was the first yeast recognized for its ability to consume pentoses in hydrolysate and convert them into polyols and ethanol (Saleh et al. 2014). Several previous studies have reported that *P. tannophilus* can produce ethanol yields from sugarcane bagasse and olive-tree pruning hydrolysate ranging from 0.004 g/g to 0.37 g/g (Cheng et al. 2007; Moya et al. 2008). Another fermentation study on hydrolysate rich in xylose, galactose, and mannose, which used *P. tannophilus*, yielded 15.9 g/L ethanol (approximately 0.31 g/g substrate) (Groves et al. 2013).

In addition to *P. tannophilus*, *K. marxianus* has received significant attention as a non-traditional, promising ethanol producer because it can ferment different sugars present in liquid hydrolysate (Mejía-Barajas et al. 2018). Some strains of *K. marxianus* have been reported to assimilate pentose sugars for growth but are incapable of converting those sugars into ethanol. Goshima et al. (2013) also reported that *K. marxianus* DMB1 could use pentose sugar for growth but did not ferment it into ethanol. However, several strains of *K. marxianus* can ferment pentose sugars into ethanol. Mueller (2009) showed that five strains of *K. marxianus* used in their research were capable of fermenting xylose to ethanol at 40°C and 45°C. *Kluyveromyces marxianus* TISTR5925 also exhibited the ability to ferment sucrose, glucose, and fructose (Murata et al. 2015).

In this study, OPEFB acid hydrolysate was fermented using *K. marxianus* and *P. tannophilus* (Figure 4). During the 72 h fermentation period, *P. tannophilus* produced more ethanol, and this concentration increased in correlation with the sugar consumption profile. In contrast, *K. marxianus* produced ethanol that remained constant throughout the duration of the fermentation process (Figure 4A). *Pachysolen tannophilus* achieved an ethanol yield of 0.035 g/g, approximately 6.79% of the theoretical ethanol yield, at 48 h of fermentation. While this was going on, *K. marxianus* produced 0.051 g/g of ethanol and 10.06% theoretical ethanol after 24 h of fermentation (Figure 4B).

Kluyveromyces marxianus produces a higher value of ethanol yield than that of *P. tannophilus* at the beginning of fermentation. However, ethanol yield sharply decreased after 24 h of fermentation. This decrease might be due to *K. marxianus* being less tolerant of inhibitory compounds in the hydrolysate. Goshima et al. (2013) showed that the growth of *K. marxianus* DMB1 and *K. marxianus* NBRC1777 was slow, resulting in low ethanol production when cultured on eucalypt plant hydrolysate. Both yeasts were less tolerant of inhibitors found in the hydrolysate.

Furfural, HMF, phenolic acid, and acetic acid are common inhibitor substances found in hydrolysate pretreatment. Inhibitor compounds may suppress yeast growth and ethanol production. Xylose can be further broken down into furfural during the physicochemical treatment process. High temperatures and low pH can accelerate this reaction. Furfural inhibits a yeast metabolic enzyme required for ethanol production. Specifically, furfural inhibits the enzyme activity of triosephosphate dehydrogenase, which likely contributes to glycolysis suppression. In general, yeasts can convert furfural into furfuryl alcohol, a less harmful molecule; however, yeast requires time to produce the necessary enzyme for furfural breakdown (Mueller 2009). The variations in productivity seen across ethanologenic yeast strains may be attributed to strain-specific properties, including glucose uptake and metabolism, stress response capabilities, and redox balance within the biomass hydrolysate (Kim 2018).

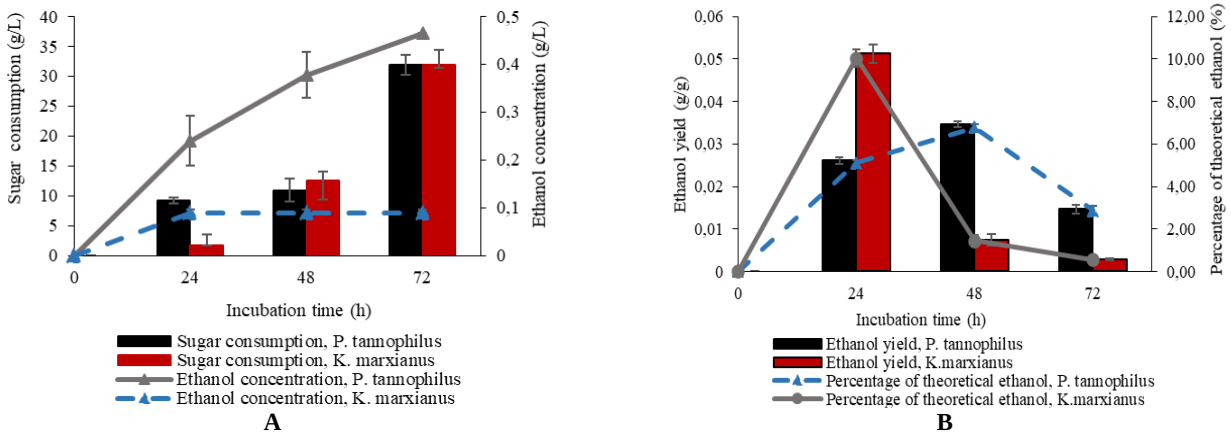

Figure 4. Sugar consumption and ethanol concentration (A), ethanol yield and the percentage of theoretical ethanol (B) on fermentation of hydrolysate pretreatment

In the hydrolysis and fermentation area, bioethanol production from OPEFB was done with oxalic acid and microwave pretreatment. Fermentation occurred both on pretreated OPEFB pulp and OPEFB acid hydrolysate. Three types of fermentation systems—SHF, SSF, and PSSF—along with two types of yeast—*S. cerevisiae* and *K. marxianus*—were used to break down and ferment pretreated OPEFB pulp. At the same time, *K. marxianus* and *P. tannophilus* were used to ferment OPEFB acid hydrolysate. It can be concluded that *K. marxianus* InaCC Y119 produced a higher ethanol yield than *S. cerevisiae* InaCC Y93 during the fermentation process of pretreated OPEFB pulp. Pre-hydrolysis before SSF is advisable, as it can reduce the fermentation duration and yield a higher ethanol concentration. *Kluyveromyces marxianus* InaCC Y119 is an excellent option for bioethanol production from lignocellulosic biomass because it can ferment both the solid component of the lignocellulosic pulp and its hydrolysate, which contains a high concentration of pentose sugar. The non-conventional yeast *K. marxianus* has been proven to be a promising ethanologenic yeast for bioethanol production. However, the wild type of this strain still has several drawbacks, such as sensitivity to high ethanol concentrations and low productivity for practical applications. All these challenges can be resolved through genetic engineering to improve the strain's suitability for the fermentation industry.

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