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Valorization of Oil Palm Empty Fruit Bunches (OPEFB) for Bioethanol Production in Indonesia

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Abstract. Indonesia, one of the world's largest producers of palm oil, generates large amounts of Oil Palm Empty Fruit Bunches (OPEFB). Its improper disposal can cause air pollution and soil acidification. OPEFB comprises lignocellulose, which can be hydrolyzed into monosaccharides to produce bioethanol. Bioethanol is a versatile product commonly produced using food crops as its raw material, but it can also be produced using lignocellulosic biomass to lessen the competition for food crops and land. Thus, this study aimed to produce bioethanol from OPEFB and enhance its production through adaptive evolution and supplementation with molasses. Adaptive evolution was carried out by exposing the yeast to ethanol concentrations of 3–12% (v/v). Native and adaptive strains are grown in OPEFB media supplemented with PEG and molasses. As a result, bioethanol was successfully produced, reaching around 10% (v/v) using a combination of OPEFB hydrolysate, PEG, and 20% molasses. Moreover, it was found that strains adapted toward 9% ethanol are more resistant to ethanol, as they can grow faster than the native strain. This shows that OPEFB can be effectively valorized for bioethanol production as a solution for OPEFB disposal and establishing a circular economy.

Keywords: adaptive evolution; bioethanol; oil palm empty fruit bunch; *Saccharomyces cerevisiae*.

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INTRODUCTION

Indonesia is one of the largest producers of palm oil and crude palm oil, contributing around 52% of global palm oil production in 2012 (Henindar & Wulan, 2020). However, 30% of the production results in oil and 70% as waste (Ishak & Ali, 2019). Oil Palm Empty Fruit Bunches (OPEFB) is the primary waste, reaching 51 million tons in 2022 (Rezki et al., 2023). The management of OPEFB is through incineration and accumulation in landfills, leading to environmental issues, such as air pollution, greenhouse gas emissions, and soil acidification (Noah, 2022).

OPEFB contains lignocellulose, which is composed of lignin (10%), cellulose (35–45%), and hemicellulose (20–40%), where cellulose and hemicellulose can be hydrolyzed by acid hydrolysis into fermentable sugars, such as glucose, fructose, and galactose (Hidayah & Wusko, 2020). These sugars can then be converted into bioethanol via fermentation. This process typically employs the yeast *Saccharomyces cerevisiae* due to its efficiency in ethanol production, which stems from its tendency to produce and accumulate ethanol to create a toxic environment for its competitors (Mahulette & Astuti, 2020; Parapouli et al., 2020). However, high ethanol concentrations cause stress to yeast and lead to growth inhibition and cell death. Thus, an adaptive evolution process is needed to increase yeast tolerance towards ethanol stress (Rustiaty, 2018).

Ethanol is a versatile organic compound used in various products, including solvents, disinfectants, alcoholic beverages, and biofuels (Alam & Tanveer, 2020). Traditionally, ethanol has been produced using food crops, but this will compete with food production and land usage; so there is a need to use non-edible feedstock, such as lignocellulosic waste (Tse et al., 2021). OPEFB, in particular, is a promising waste feedstock because it is low in cost, abundant, and difficult to dispose of (Purwoko et al., 2022). Its use in the production of bioethanol will enable both cost-

efficient bioethanol production and the sustainable disposal of this waste. Furthermore, supplementation with other carbon-rich waste, like molasses, has the potential to enhance ethanol production and yeast growth. Molasses is a byproduct of sugar crystallization that can support fermentation since it contains sugars, minerals, and vitamins essential for cell growth (Jamir et al., 2021). It also contains salts, which provide buffering capacity for maintaining optimal fermentation pH (Hurtado et al., 2021). Thus, its supplementation into OPEFB hydrolysate is expected to increase *S. cerevisiae* growth and ethanol production.

This study aims to utilize OPEFB for producing bioethanol and to enhance its production by employing an adaptive evolution method for *S. cerevisiae* and supplementation with molasses. This investigation of combination feedstocks from the lignocellulosic waste OPEFB and molasses has not been explored before (Oke et al., 2016). Hence, this study advances scientific knowledge by confirming the potential of OPEFB and molasses as feedstock for efficient ethanol production and creating a more ethanol-resistant *S. cerevisiae* using adaptive evolution. This research will also contribute to establishing a circular economy that uses waste to drive economic growth in society and prevent pollution caused by improper OPEFB waste disposal.

METHODS

Time and Location

This research was conducted from April 2024 to August 2024 and took place in the laboratory of Indonesia International Institute for Life Sciences (i3L) in Jakarta.

Ethanol Production

Pre-treatment of Oil Palm Empty Fruit Bunches (OPEFB)

Firstly, OPEFB was cut, washed, dried, blended, and mixed into 2% H_2SO_4 (Merck) with a ratio of 1:10 (OPEFB: acid) in a tightly closed 500-mL Duran bottle (Pyrex). Then it was autoclaved for 1 hour at 12°C using SX-700 Autoclave High-Pressure Steam Sterilizer. Then, the hydrolysate was neutralized using $Ca(OH)_2$ (Merck) until pH reached 5.5 using pH meter ST350 Ohaus. After that, OPEFB was filtered to remove the salt with Whatman No. 1 filter paper (Cytiva) and autoclaved at 121°C for 15 minutes (Chandrasiri et al., 2022).

Analyzing Sugar Levels in Hydrolysate

The sugar levels in the hydrolysate before fermentation were analyzed using High-Performance Liquid Chromatography (HPLC). This procedure was carried out with Thermo Scientific Dionex UltiMate 3000 with Shodex SUGAR SP0810 column. Analysis was carried out with a column temperature of 70°C and its mobile phase used deionized water. The Refractor Index (RI) detector was set at a temperature of 50°C, with a flow rate of 0.6 mL/min. Results were compared with standard curves of glucose, fructose, galactose, sucrose, arabinose, and xylose sugars to determine their concentrations (Seong et al., 2016).

Adaptation of Saccharomyces cerevisiae to Ethanol

After that, *S. cerevisiae* (FNCC 3012) obtained from Food and Nutrition Culture Collection *Universitas Gadjah Mada* (FNCC UGM) was grown in Yeast Extract-Peptone-Dextrose (YPD) liquid media from Merck, and then its morphology was confirmed by gram staining and microscope observations. The culture of *S. cerevisiae* that had been grown was then streaked on YPD agar using the quadrant streak method to be used as stock.

For adaptive evolution, yeast that has been grown on agar media was transferred to YPD media as pre-culture. The pre-culture was incubated at 30°C for 24 hours and transferred to 5 YPD media that had been added with 0%, 3%, 6%, 9%, and 12% (v/v) ethanol. Adaptation data were obtained by sampling every 4 hours for 24 hours and at 48 hours. Optical Density (OD), pH, and viable cell count (CFU/mL) of samples were measured to create a growth curve. OD data were obtained by measuring absorbance with Infinite M200 NanoQuant Plate Reader at 600 nm; pH data were measured with a universal pH indicator; and viable cell count was measured by the Miles-Misra method (Mavrommati et al., 2023).

Adaptive culture was streaked on YPD agar as stock. From the stock, it was regrown in preculture, inoculated into YPD media, and samples were taken every 24 hours for 5 days. Samples were analyzed for their ethanol production with Gas Chromatography Trace 1310. Before ethanol was injected into the gas chromatography machine, samples were filtered with a 0.22 μm syringe filter (Sartorius) into a gas chromatography vial. Ethanol standards were made using absolute ethanol (Merck) diluted with deionized water to concentrations of 0.05; 0.1; 0.2;

0.4; 0.6; and 1% (v/v) ethanol. From the results, adaptation results of 0% and 9% were used for ethanol growth using the prepared OPEFB media (Riccardino & Cojocariu, 2020).

Bioethanol Production and Purification

OPEFB media was added with 0.25% (w/v) $(NH_4)_2SO_4$ (Smartlab), 0.1% (w/v) KH_2PO_4 (Himedia), 0.5% (w/v) $MgSO_4$, 0.25% (w/v) yeast extract (Merck). In addition, OPEFB media was given different treatments, namely: OPEFB without additional supplementation, OPEFB with the addition of polyethylene glycol (PEG), OPEFB with the addition of PEG and 10% (v/v) molasses, and OPEFB with the addition of PEG and 20% (v/v) molasses. Molasses were added before the media was autoclaved, while 0.125 g/mL PEG 400 (Merck) was added after autoclaving (Liu et al., 2014).

The OPEFB media was then inoculated with 0% native yeast preculture and 9% adaptive yeast preculture with 5% inoculum. Growth was carried out for 5 days at 30℃, with sampling every 4 hours for the first 2 days and every 24 hours thereafter. Data obtained from samples were used to calculate pH and CFU/mL to determine the yeast growth curve. Samples taken every 24 hours were used for ethanol content analysis using gas chromatography (Iram et al., 2018).

The ethanol production results were separated using simple distillation. The distillation temperature was slowly increased to 60–70℃, and results at this temperature were discarded. The temperature was then increased to 79–85℃ for ethanol extraction (Spaho, 2017).

Gas Chromatography

Bioethanol levels were checked using Gas Chromatography Trace 1310. Around 800 µL of fermentation broth was centrifuged and separated from yeast cell sediment. Supernatant samples and standards were filtered with a 0.22 µm syringe filter (Sartorius) into a gas chromatography vial. Approximately 1 µL of each sample and standard were inserted into the gas chromatography machine, then the temperature was set at 40℃ isothermally for 4 minutes, the inlet temperature at 150℃ with split mode using the ratio of 20:1, and carrier gas was helium with a constant flow of 1 mL/min. The temperature was then raised to 120℃ for Flame Ionization Detector (FID) results. Results were obtained in the form of a chromatography curve. From the analysis of the chromatography curve, the concentration of ethanol produced between adaptive and native strains will be compared (Riccardino & Cojocariu, 2020).

RESULTS AND DISCUSSION

Sugar Content in OPEFB Hydrolysate

The sugar content of OPEFB hydrolysate was first analyzed. As previously mentioned, OPEFB is a lignocellulosic biomass, typically composed of lignin, cellulose, and hemicellulose (Hidayah & Wusko, 2020). Cellulose is the primary polysaccharide, which is made up of glucose units linked by β-1,4-glycosidic bonds. Hemicelluloses are polysaccharides made up of monosaccharides, such as glucose, galactose, mannose, arabinose, and xylose; and uronic acid groups, such as galacturonic acid and glucuronic acid (Świątek et al., 2020). On the other hand, lignin is a macromolecule that keeps the lignocellulose matrix together. The glycosidic bonds of cellulose and hemicellulose need to be hydrolyzed to release the monosaccharides, which can be done by acid hydrolysis using sulfuric acid (Zhou et al., 2021). The monosaccharides can then be used by microbes to create ethanol (Maitan-Alfenas et al., 2015). The resulting concentration of monosaccharides from the OPEFB hydrolysate is shown below in Table 1.

Table 1. Composition of monosaccharides in neutralized OPEFB hydrolysate

Monosaccharide	Concentration (g/L)
Glucose	1.848
Arabinose	3.841
Xylose	6.245

It was found that there is a higher content of pentose sugars, which are 3.841 g/L arabinose and 6.245 g/L xylose, compared to the hexose sugar, which is 1.848 g/L glucose. However, *S. cerevisiae* can only ferment hexose sugars (Ruchala et al., 2020). Hence, the OPEFB media was supplemented with molasses, which is rich in sucrose (30–35%) as well as glucose and fructose (10–25%), as an additional carbon source derived from waste (Jamir et al., 2021).

Adaptive Evolution

Adaptive evolution is a change of phenotype due to an inherited change when facing certain environmental conditions to increase the survivability of the organism (Edelaar et al., 2023). For *Saccharomyces cerevisiae*, adaptive evolution was done to increase ethanol tolerance, as production of ethanol usually results in ethanol

concentrations of 15% (v/v), where most of the yeast would not survive. Thus, adaptive evolution—by acclimatizing yeast to different ethanol concentrations—was needed to ensure the survival of yeast in higher ethanol concentrations (Zhang et al., 2019). The result of adaptive evolution was obtained as a growth curve shown in Figure 1. Ethanol is produced in the log phase since it is a primary metabolite, although its production still continues until the stationary phase (da Silva et al., 2018). Thus, observation of the growth curve prioritizes the log phase, where sampling was conducted at 4-hour intervals for 24 hours and the 48th hour.

In the growth curve of the native strain grown in 0% ethanol (Figure 1), the microbe immediately enters the log phase from 0 to 16 hours, followed by a stationary phase until the end of the 48-hour observation period. Adaptive strains 3 and 6% have a short lag phase from 0–4 hours. Meanwhile, the 9% strain has an extended lag phase from 0–8 hours, followed by a log phase up to the 20th hour. The 12% strain has the longest lag phase, up to the 16th hour, followed by a short log phase until the 20th hour, as well as a stationary and death phase. Based on the results, the strain that grows best in the highest ethanol concentration is the 9% strain, resulting in a peak OD and viable cell count value similar to the native strain. As such, this strain was used for further analysis.

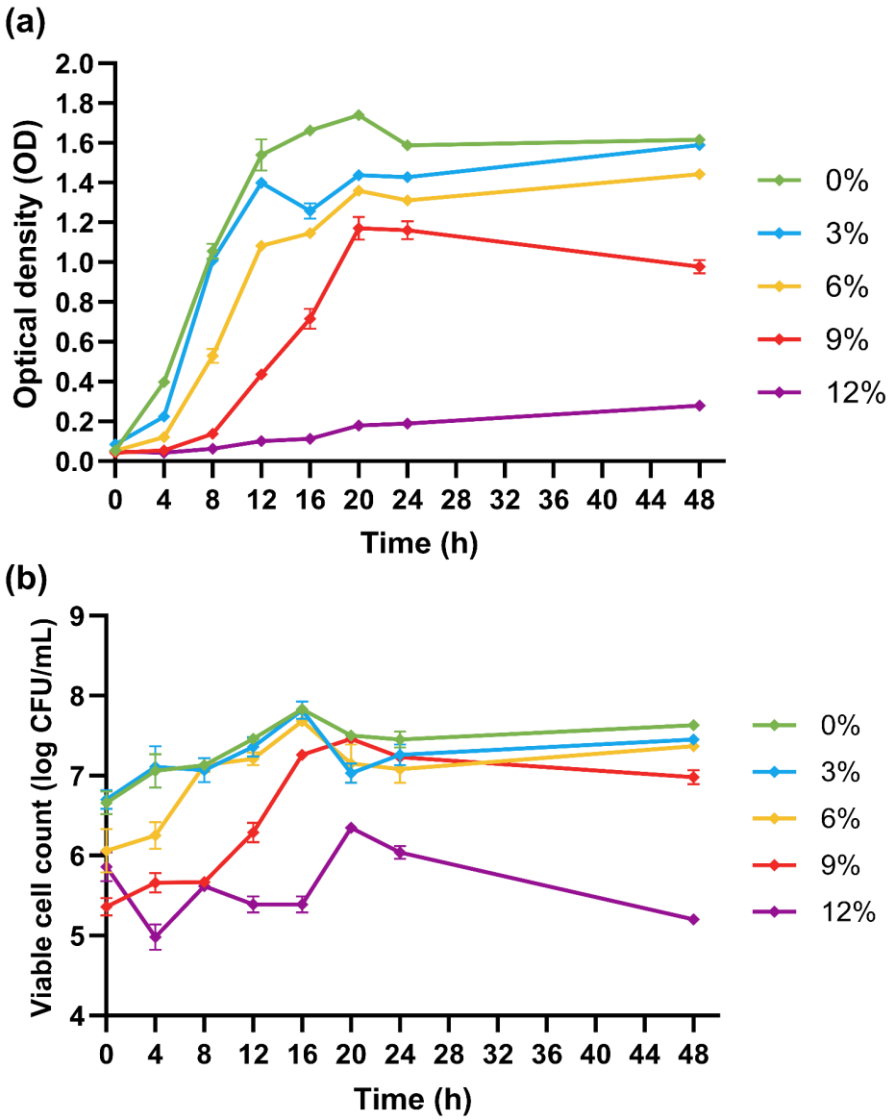

Figure 1. Adaptive evolution growth curve based on (a) Optical Density (OD) and (b) viable cell count (CFU/mL) in various ethanol concentrations (0, 3, 6, 9, and 12%).

Bioethanol

Ethanol is an alcohol produced by *Saccharomyces cerevisiae* from the fermentation of hexose sugars under anaerobic conditions (Ruchala et al., 2020). Analysis of ethanol production by the native and adaptive strains in YPD media shown in Figure 2 proved that ethanol production of the adaptive strain is faster than the native strain, with the adaptive strain reaching 0.34% (v/v) ethanol at 96 h, while the native strain only reached 0.275%. In a study by Rustiaty (2018), the adaptive strain also showed a higher ethanol content compared to the native strain. There is a unique occurrence in the adaptive strain data where there is an ethanol decrease after 120 h, which could be due to *S. cerevisiae*'s tendency to consume ethanol when lacking a carbon source (Tomova et al., 2019). This is consistent with the OPEFB culture ethanol data, where cultures supplemented with 0–10% molasses had a decrease in ethanol content, but not those supplemented with 20% molasses.

The growth and ethanol production of these strains were then tested on OPEFB media with various supplementations. Results are shown in Figures 3 and 4.

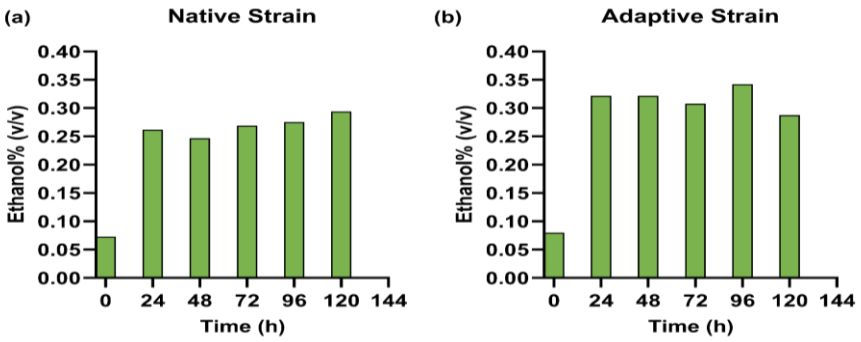

Figure 2. Ethanol production of native (a) and adaptive strains (b) in YPD media at different incubation times (0, 24, 48, 72, 96, and 120 hours).

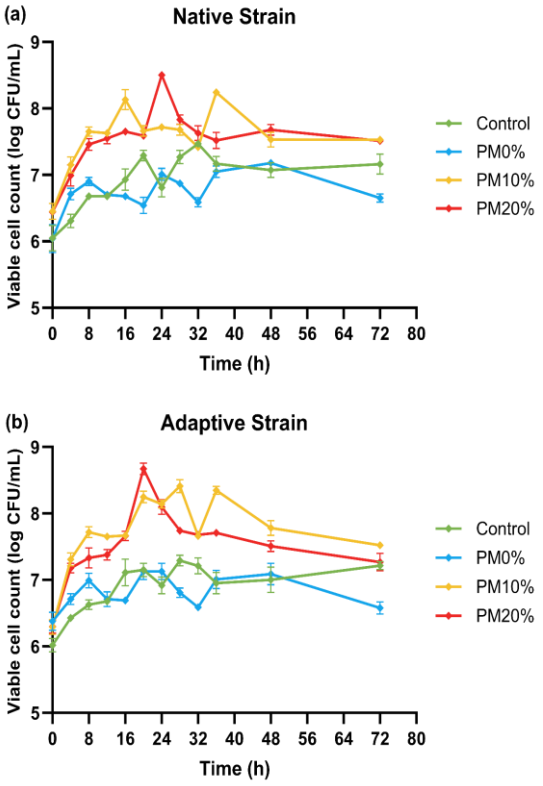

Figure 3. Growth curve of native (a) and adaptive strains (b) in OPEFB media with various supplementations: control, PEG (PM0%), PEG and 10% molasses (PM10%), as well as PEG and 20% molasses (PM20%).

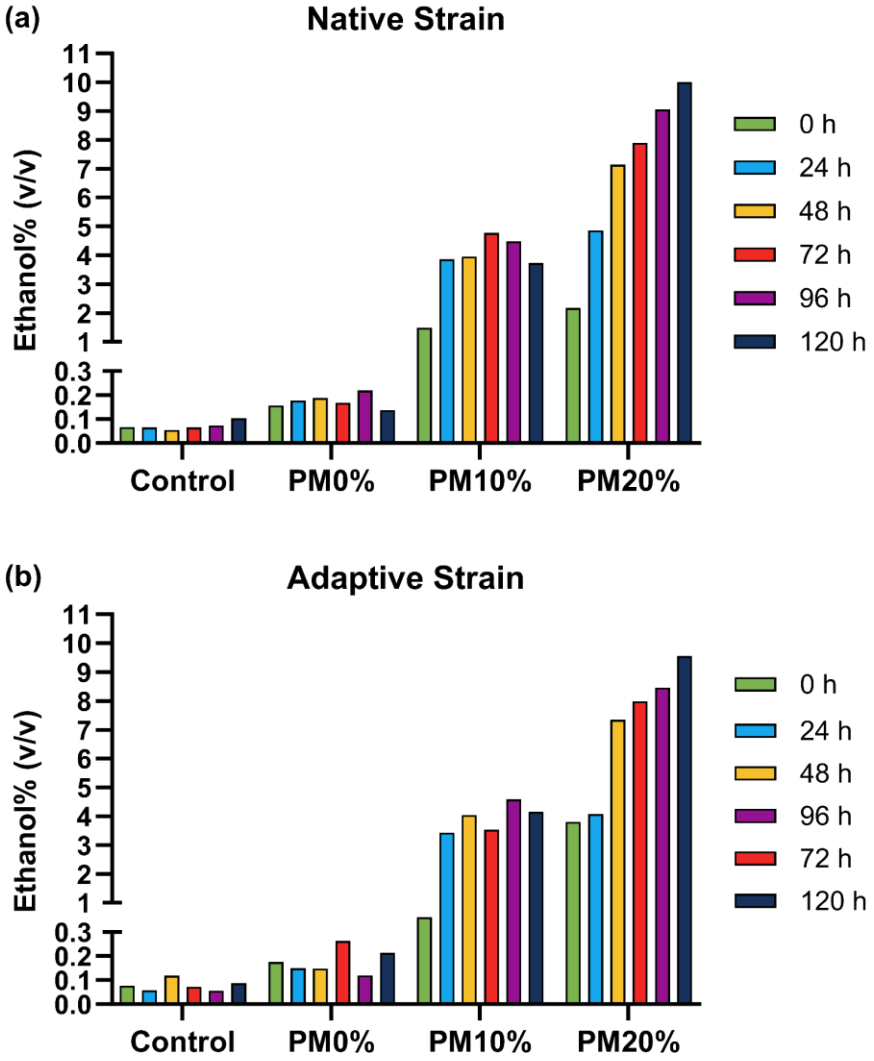

Figure 4. Ethanol production of native (a) and adaptive strains (b) in OPEFB media with various supplementations: control, PEG (PM0%), PEG and 10% molasses (PM10%), as well as PEG and 20% molasses (PM20%) at different incubation times (0, 24, 48, 96, 72, and 120 hours).

Figures 3 and 4 showed that detoxification using PEG increases the growth of the yeast in the first 8 hours, along with ethanol production. Similarly, in a study by da Costa Nogueira et al. (2021b), the addition of 0.25 g/mL PEG 400 doubled cell viability and increased ethanol production. This is because PEG detoxifies furan and phenolic inhibitors in the hydrolysate, where the oxygen atom of PEG interacts with the hydroxyl group of the inhibitors, preventing contact of inhibitors with the cell wall (da Costa Nogueira et al., 2021a).

According to Figure 3, *S. cerevisiae* grown in PEG and molasses 20% has the highest viable cell count. The native strain reached the peak of the log phase at 24 h, while the adaptive strains reached the peak at 20 h, indicating that the adaptive strain has faster growth. Based on Rustiaty et al. (2018), adaptive yeast could accelerate cell growth, especially at high ethanol content. A similar result is observed in Figure 4, which shows that the yeast grown in PEG and molasses 20% produces the highest ethanol content, reaching around 10% (v/v) for both native and adaptive strains. In comparison, Pandey et al. (2022) obtained 12.31% (v/v) ethanol content using 40% (v/v) molasses. Hence, the results show that adaptation increases the yeast's resistance to ethanol but could not contribute significantly to ethanol production.

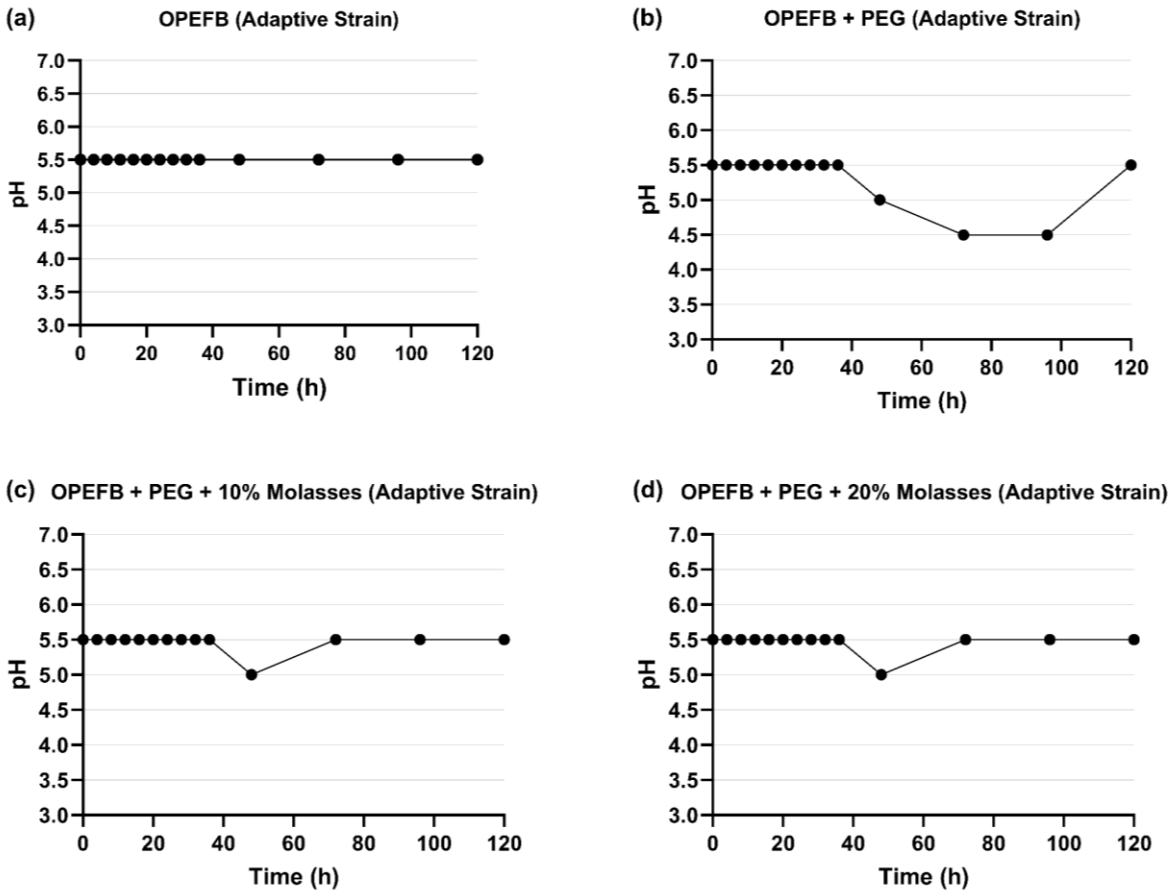

Figure 5. pH curve of adaptive strains.

The positive effect of molasses is due to its buffering capacity from its salt content, allowing it to maintain optimal pH for fermentation (Hurtado et al., 2021). This phenomenon was seen in the pH of the adaptive culture supplemented with molasses in Figure 5, which only dropped to pH 5 and increased back to pH 5.5, whereas the culture without molasses decreased in pH to 4.5 after 72 hours. Molasses' beneficial effect on microbial growth and ethanol production can also be due to their vitamin contents, allowing the energy needed for vitamin production to be allocated for cell growth instead (Jamir et al., 2021; van Dijk et al., 2020). Furthermore, molasses contains minerals (e.g., potassium, phosphorus, and magnesium) that are required for cell growth and as cofactors for enzymes in metabolism, including glycolysis and ethanol fermentation (Utami et al., 2014; Li et al., 2020).

Ethanol produced from OPEFB media supplemented with PEG and 20% molasses has been purified through distillation and analyzed. The resulting yield, purity, and recovery percentages are shown in Table 2.

Table 2. Yield, purity, and recovery percentage of distilled OPEFB culture supplemented with PEG and molasses 20%.

	Yield (%)	**Purity (%)**	**Recovery (%)**
Native strain	19.333	37.309	72.123
Adaptive strain	12.667	52.567	69.729

Distillation of fermentation broth from OPEFB culture supplemented with PEG and molasses 20% after 120-hour incubation produced 37-53% (v/v) ethanol. However, this purity is still quite low, as the maximum purity that could be achieved through distillation is 95%. Low purity could be due to temperature fluctuations during heating. Moreover, the fractional distillation method is preferably used when the difference in boiling point is less than 25°C, such as in this case with ethanol (78.5°C) and water (100°C) (Mchedlov-Petrossyan, 2020). A similar yield of 45% is acquired by Ridwan et al. (2023), but increased to 90% by further distillation. In future research, it is suggested to use a temperature regulator, fractional distillation method, or

repetition of distillation to gain a higher bioethanol purity. Nevertheless, conventional distillation can only reach 95.57% (w/w) purity since ethanol forms azeotrope with water, resulting in the same boiling point. Hence, for the production of fuel-grade ethanol with 99.5% (v/v) purity, further purification needs to be conducted through azeotropic distillation, extractive distillation, adsorption methods, or membrane technology (Ibrahim et al., 2022; Mekala et al., 2022).

Future studies should focus on improving the distillation process for extracting ethanol from OPEFB to increase its purity. Furthermore, optimization of fermentation conditions, like pH, temperature, and nutrient levels, may help to increase yields even further (Altınışık et al., 2024). In addition, genetically altering or metabolically engineering *Saccharomyces cerevisiae* could considerably increase bioethanol production efficiency (Kembaren, 2023). These improvements would encourage the long-term use of OPEFB with the supplementation of molasses as feedstock for bioethanol production.

This study presents the feasibility of using OPEFB and molasses as combination feedstocks for efficient bioethanol production, which has not been investigated in previous studies (Oke et al., 2016). Unlike most studies, this research further explores the distillation of ethanol, which would be crucial for further downstream processing (da Costa Nogueira et al., 2021a; Pandey et al., 2022). As such, this study contributes to scientific development by confirming the feasibility of OPEFB and molasses supplementation for efficient ethanol production and presents a more ethanol-resistant *S. cerevisiae*. This research benefits society by promoting a circular economy, which can boost economic growth, and improve the environment by preventing pollution due to improper OPEFB waste disposal.

CONCLUSION

This study successfully demonstrates the potential of OPEFB as a raw material for bioethanol production as well as the adaptive evolution of the yeast *S. cerevisiae* and the addition of molasses for enhancing bioethanol production. Through this research, approximately 10% (v/v) of bioethanol was successfully produced using OPEFB hydrolysate and supplementation of PEG and 20% molasses after 120 hours of incubation. Furthermore, it was found that *S. cerevisiae* adapted to 9% ethanol is more resistant to ethanol, as they grow much faster than the native strain. Therefore, it can be concluded that OPEFB with molasses supplementation can serve as an alternative carbon source for bioethanol production and also promote a more sustainable waste management of OPEFB. However, the production of bioethanol using this feedstock can be further enhanced by optimization of fermentation conditions or exploration of purification methods.

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