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Cocoa Pod Husk (CPH) for Biomass on Bioethanol Production

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***Abstract*— Cocoa pod husk (CPH) is one of underutilized agricultural lignocellulosic biomass. CPH and its contents are considered suitable to be used as raw material in the production of 2G biofuels. Various hydrolysis methods and processing are still being developed and have not yet been well studied. This study aimed to investigate the efficiency of hydrolysis process using microwave irradiation and the stress tolerance phenotype of yeast. The pretreatment of CPH biomass was carried out using hydrogen peroxide (H_2O_2). The chemical and structural component of CPH was analyzed before and after pretreatment. The results showed that H_2O_2 reduced up to 34% of lignin and increased the solubility of this component. Pretreated CPH powder was hydrolyzed by combining 1M H_2SO_4 and microwave-assisted hydrolysis, resulting in high glucose-xylose concentration within short reaction times. The highest glucose-xylose concentrations from CPH were 3.40 g/L – 0.94 g/L within 12 minutes at 180 °C. The oxidative-fermentative test showed that yeast Y003 could ferment xylose. The stress tolerance test showed that *S. cerevisiae* Y003 tolerance up to 40 °C temperature, up to 14% ethanol concentration, and up to pH 3 under pH stress. The highest glucose-xylose consumption by *S. cerevisiae* Y003 was observed under the treatment of E2 (93.59%), with ethanol production up to 1.85 g/L. Fermentation efficiency was 85 – 97%, indicating that this research succeeded in producing bioethanol from CPH biomass. Cocoa pod husk is a promising resource for further development of bioethanol production.**

***Keywords*— Pretreatment; hydrolysis; microwave heating; fermentation-related stresses; ethanol.**

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I. INTRODUCTION

A significant increase in fossil fuel consumption has affected the rapid depletion of global fossil fuel reserves and contributed to the high rate of global climate change [1]. The International Energy Agency (IEA) stated that in 2022, total global carbon dioxide (CO_2) emissions are on course to reach around 36.8 Gt [2]. CO_2 contributes significantly to global warming due to the burning of transportation fuels, along is reported to have affected the quality of human life, accounting for a major part of environmental pollution. Therefore, this problem has prompted researchers to explore and investigate alternative fuel sources which are environmentally safe and eco-friendly [3], [4].

Biofuels are gaining global attention as a sustainable and renewable alternative energy resource that can lower the demand for crude oil and gasoline. Biofuels can be produced indirectly from wastes, agricultural residues, agricultural products, and living plants [5]. Biofuels are a promising energy resource, and the demand for these biomass-derived fuels increased in recent years [6]. Renewable biofuels can be gas or solid forms such as biogas, syngas, and wood pellets; moreover, they can also include liquid forms like biodiesel, bioethanol, bio-oil, and biobutanol [7].

The most common biofuels are bioethanol and biodiesel, used instead of regular gasoline and diesel. Bioethanol is converted from biomass to ethanol through fermentation and can be produced from carbohydrates and cellulosic biomass. Therefore, biodiesel is converted from fats or vegetable oils by esterification and transesterification [8]. The stages of bioethanol production begin with extracting sugars from the raw materials, converting the sugars into ethanol by fermentation, and then distilling the resulting ethanol to purify

bioethanol. The worldwide bioethanol production reached 27.2 million gallons in 2021 [9], [10].

Raw materials are the main thing in alternative energy fuels production. Biofuels derived from formerly edible feedstocks, known as first generation (1G). 1G biofuel has certain negative impacts as they directly compete with agricultural land, promote deforestation and cause the loss of native species [11], [12]. The development of biofuel technology has led scientists to find new sources of biomass that do not require new land for plant growth. Second generation (2G) has the advantage of being able to produce biofuel using non-edible raw materials and agricultural residues, including lignocellulose [13]–[16]. Converting lignocellulosic biomass into bioethanol involves several steps as pretreatment, hydrolysis, and fermentation steps. Various chemical and physical treatments are required in converting lignocellulose liquid fuels [17]. Pretreatment includes separating each lignocellulosic content to improve access to cellulose and hemicellulose through hydrolysis. During hydrolysis, carbohydrates are converted into various sugar monomers with aliments from enzymes or acid catalysts, also known as saccharification. The converted monomers are then transformed into ethanol and other by-products through fermentation, which is acted upon by microorganisms such as bacteria and yeast [18], [19].

Cocoa pod husks (CPH) account for approximately 80% of cocoa farming residues that occur during the initial processing steps of cocoa beans [20], [21]. Over the past 40 years, cocoa production has increased steadily, and several important benefits to sustainable production practices have been observed. Cocoa (*Theobroma cacao* L.) production in 2021 reached about 4.8 million tons worldwide. In Asia, Indonesia is the largest producing country [22], [23]. One ton of dry cocoa beans will produce 10 tons of wet shells. Pod husks waste is generally left to rot in cocoa plantations, causing social and environmental concerns [24]–[26].

The high concentration of lignin compounds in CPH may influence the rate of the hydrolysis process. Therefore, it is necessary to pretreat CPH before the fermentation process. Lignocellulosic pretreatment can be carried out chemically with H_2SO_4, H_2O_2, or NaOH. Previous research on the pretreatment of CPH with 6% NaOH reduced lignin content from 30.46% to 24.64% (w/w). Corn straws pretreated with hydrogen peroxide reduced the composition of lignin, cellulose, and hemicellulose components by 19.6, 32.8, and 6.2% [27], [28]. The CPH pretreatment process with H_2SO_4 and H_2O_2 showed hydrogen peroxide is more efficient in lignin delignification in CPH, whereas H_2O_2 can reduce 71.34% of total lignin content and increase 39% of cellulose [29].

Organic compounds such as cellulose and hemicellulose cannot be converted to ethanol by commercial yeast. Further processing must be done before the fermentation stage. The hydrolysis process helps break down hemicellulose and cellulose compounds into sugar monomers. Microwave heating can accelerate the cellulose and hemicellulose compounds hydrolyzed and increase the total amount of reducing sugars [30]. Microwave heating has been widely used for hydrolysis due to often obtaining higher yields in short reaction times. A previous study reported that the longest for starch degradation using microwave heating was 10 min [31]. However, the CPH hydrolysis using microwave heating needs further observation.

Yeast *Saccharomyces cerevisiae* was commonly used in industrial fermentation. During fermentation, yeast received various stresses, such as high ethanol stress, temperature stress, and hyperosmotic stress, leading to the depletion of ethanol production [6], [32]. Therefore, it is important to know the optimal conditions for yeast growth. This research aims to evaluate the stress tolerance phenotype of yeast and perform fermentative activity analysis based on sugar consumption and ethanol production. This study also sought to evaluate hydrolysis efficiency using microwave-assisted hydrolysis to enhance the sugar concentrations in CPH hydrolysate.

II. Materials and Method

All experiments in this study were performed in triplicate, and values are presented as mean ± standard deviation (SD). All chemicals were of analytical grade. Fig. 1 shows the flow of the research.

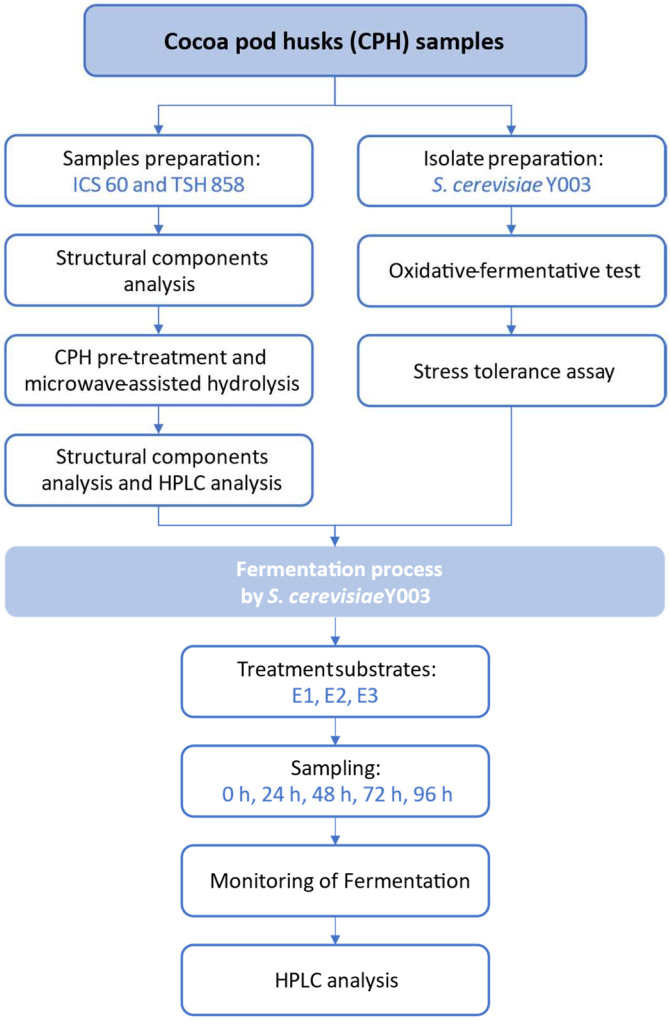

Fig. 1 Research Flowchart.

A. Materials

The pod husks of the cocoa plant (*Theobroma cacao* L.) used in this study were sourced from Payakumbuh (West Sumatra), Indonesia, with cellulose as the main component (34. 23% ±1.06). Two varieties were obtained in this study,

TSH 858 and ICS 60. The chemicals used were hydrogen peroxide, sulfuric acid, sodium hydroxide, and ethanol. Several pieces of equipment used in this study, such as an incubator shaker, centrifuge (Sigma Laborzentrifugen, Osterode am Harz, Germany), digestion microwave (Milestone START D, Milestone Inc, Bergamo, Italy), high-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan), and vacuum filtration (Rocker 300 – MF 31, Rocker, Kaohsiung, Taiwan).

B. Sample collection

The selected pods were washed to remove soil, sand, and dirt. The pods were then shredded into pieces, and the beans were separated. The remaining pod husks were further chopped and dried at 80 °C until a constant dry weight was achieved. The dried CPH was then ground and sieved to obtain a powder with a mesh size of 0.149 mm according to standard methods [29], [33]. The structural components of untreated CPH were then analyzed. All samples were stored in a refrigerator at 4 °C until further use.

C. Pretreatment and Microwave-assisted Hydrolysis of CPH powder

1) Pretreatment with H_2O_2: The pretreatment of CPH powder was performed with hydrogen peroxide (H_2O_2) based on earlier methods with some modifications [34]. 200 g of dry mass was suspended in 7.5% (v/v) hydrogen peroxide (1 g/9 mL) and incubated for 4 hours at room temperature. The mixture was then rinsed with distilled water until the pH was neutral. The mixture was then dried in a drying oven at 80 °C for 72 hours. Determination of total lignin, cellulose, and hemicellulose was performed on CPH-pretreated samples.

2) Hydrolysis of pretreated CPH powder by Microwave Heating: Hydrolysis of pretreated samples was carried out by microwave-assisted hydrolysis based on the modification of earlier methods [35]. The pretreated CPH powder (2 g) was suspended in the prescribed concentration of 5% (v/v) sulfuric acid solution (10 mL) and put in 100 mL Teflon® tube. The sealed tube equipped with a stirrer was heated at 1000 W using a digestion microwave (Milestone START D, Milestone Inc, Bergamo, Italy) with a thermocouple inserted into the reactor to control the temperature during the heating process. Hydrolysis is performed at various temperatures ranging from 160 °C to 200 °C with 12 min irradiation time and a monitor connected to the microwave to observe the heating process. The heating was maintained with 12 min of initial heating and 10 min of cooling.

After the reaction process, the Teflon tube was immediately cooled in an ice bath for 20 to 30 minutes. The hydrolysate was filtered with Whatman filter papers No. 40 using vacuum filtration (Rocker 300 – MF 31, Rocker, Kaohsiung, Taiwan) 110V/60 Hz to separate the solid and the liquid. Afterward, the liquid fraction was collected, and pH was adjusted until the pH was 5.5±0.2 with NaOH pellet (Merck, Darmstadt, Germany), which was measured using a pH meter. Aliquots (500 µL) of the sample solution were transferred into a centrifuge tube and centrifuged at 14,000 ×g for 15 min to precipitate the solids. The supernatant was filtered and analyzed for sugar concentration. The pretreated CPH liquid was stored in a refrigerator at 10 °C for further usage.

D. Analysis of Glucose-xylose Concentration

The liquid concentrations of glucose and xylose were determined using HPLC (Shimadzu, Kyoto, Japan) equipped with a Bio-Rad (Hercules, CA) Aminex HPX87H column (300-7.8 mm). The column was run at 60 °C using a RID-10A refractive index detector. The mobile phase was 5.0 mM H_2SO_4 at a rate of 0.6 ml/min [36]. All samples were centrifuged at 14,000 ×g for 15 min to remove the cell mass and other water-insoluble material and filtered through a 0.22 µm filter before analysis.

E. Isolate Preparation

The isolate used in this study was *Saccharomyces cerevisiae* Y003 obtained from the Biotechnology Research Center, which was maintained in 20% (w/w) glycerol stock. This isolate was recovered using yeast peptone dextrose (YPD) plate medium (1% yeast extract, 2% peptone, 2% glucose). Cultures were aseptically streaked on YPD plate media and incubated at 30 °C for 48 hours.

F. Oxidative-fermentative test

This test was performed using a Hugh and Leifson's OF basal medium containing a single sugar composition (2% peptone, 5% sodium chloride, 10% xylose, 0.3% dipotassium phosphate, 3% agar, 0.03% bromthymol blue) in 1 L distilled water. Adjusted the pH medium to 7.1 prior to autoclave. Afterward, sterile-filtered 10 % xylose solution was added aseptically. The sterile medium was aliquoted into sterile test tubes and cooled unslanted. The yeast isolate was inoculated by stabbing tubes containing an OF medium and incubated at 30 °C for 48 hours. Test results were qualitatively determined by a change in medium color, indicating the pH medium's decrease[37].

G. Stress Tolerance Assay

The stress tolerance assay of *S. cerevisiae* Y003 isolate was performed by a simple spot assay method in media containing various stress conditions based on the previous method [38]. As the main culture, *S. cerevisiae* Y003 was cultured in a YPD medium and incubated at 30 °C for 24 hours. Serial dilutions were then performed. Approximately 3 µL of culture from each dilution was spotted on YPD plate media containing various concentrations of ethanol (6%, 8%, 10%, 12%, and 14%) and pH 3, pH 4, and pH 5 of YPD plate medium. Various incubation temperatures (30 °C, 37 °C, and 40 °C) were then performed to assess temperature tolerance. The experiment was performed in triplicate over 72 hours of observation.

H. Fermentation by S. cerevisiae

As the main culture, *S. cerevisiae* Y003 was grown in YPD preculture media until OD_{600nm} = 0.8-1. Approximately 5% of the inoculum was moved to a 200 mL Erlenmeyer flask containing fresh liquid YPD medium and incubated at 30 °C with a shaking incubator at 150 rpm. Cells were harvested by centrifugation at 4 °C and 14,000 ×g for 10 min and then resuspended in 0.9% NaCl.

Fermentation was conducted based on earlier methods with some modifications [39]. Fermentations were performed with

50 mL of fermentation filtrate in 100 mL Erlenmeyer flask containing various concentrations of fermentation substrate and inoculated with 10% (v/v) of yeast inoculum. Fermentation media variations: E1: (hydrolysate liquid); E2: (hydrolysate liquid + 1% yeast extract + 2% peptone); E3: (hydrolysate liquid + 1% yeast extract + 2% peptone + 2% glucose + 2% xylose). All fermentation substrates were sterilized using a 0.22 µm filters. Fermentation occurred in a closed system with shaking 150 rpm for 96 hours in a shaking incubator at 30 °C. Each fermentation was performed in triplicate to analyze the fermentation parameters and sugar consumption.

I. Monitoring of Fermentation

Average concentrations of ethanol and sugars produced were determined using HPLC at the time of sampling. During the first 48 h of fermentation, samples were taken every 24 h to analyze the fermentation parameters, and every 24 h until 96 h of fermentation to determine sugars consumption by yeast Y003 isolate. The kinetic parameters of fermentation were calculated to determine the efficiency of ethanol, productivity of ethanol and ethanol yield. Fermentation efficiency was obtained from the ratio of the average actual produced ethanol to the theoretically produced ethanol. Determination of ethanol yield was calculated from the ratio between produced ethanol and sugars consumed by yeast cells during fermentation. Ethanol productivity is a comparison between the ethanol produced and fermentation time. The total of sugars consumed was calculated from the amount of initial sugar concentration decreased by final sugar concentration.

III. Results and Discussion

A. Structural Components and Hydrolysis of CPH powder

CPH chemical composition analysis was performed to compare the CPH chemical composition before and after pretreatment. Characterization of pretreated CPH revealed a 28.23% lower lignin level in CPH TSH 858 substrate (Table 1). At the same time, total lignin in CPH ICS 60 reduced by 34.63%. The cellulose component increases slightly, ranging from 35.27 to 37.66%, hemicellulose decreases, and the final amount ranges from 6.85 to 8.94%. The hemicellulose, cellulose, and lignin compositions in untreated and pretreated CPH are close to previous investigations obtained from the same part of cocoa and structural characterization of biomass. The cellulose component obtained was similar to Dahunsi *et al.* [34] where the final total cellulose was reported to range from 33.97 to 36.63%.

TABLE I
Structural Components of CPH Powder Before and After the Pretreatment with Hydrogen Peroxide (H_2O_2)

Sample	Hemicellulose (%)	Cellulose (%)	Lignin (%)
TSH 858 untreated	10.30	34.98	30.32
TSH 858 pretreated	8.94	35.27	21.76
ICS 60 untreated	17.36	33.48	31.39
ICS 60 pretreated	6.85	37.66	20.52

Using H_2O_2 as a pretreatment agent had a positive effect in terms of structural solubilization. In this study, hydrogen peroxide resulted high solubilization of the lignin components due to a decrease in lignin levels through the degradation of lignin polymers [29]. According to Cai *et al.* [27] hydrogen peroxide can reduce 19.6% lignin in pretreated corn straw. Another study reported that the use of alkalis in different biomass substrates completely flattened important peaks associated with lignin prior to the anaerobic digestion process [40-42].

Hydrolysis was done to degrade polysaccharide polymers such as cellulose and hemicellulose on the pretreated CPH substrate into sugar monomers. The hydrolysis process results using microwave heating (Fig. 2) showed that the highest glucose and xylose concentrations were treated at 180 °C with concentrations of glucose – xylose were 3.40 g/L and 0.94 g/L. The results show increased substrate sugar concentration after hydrolyzing using a combination of acid and microwave. Kuroda *et al.* [35] was reported that combination of microwave heating for 12 minutes with additions acidic ILs solution such as H_2SO_4 and HSO_4 synergistically enhanced the hydrolysis of cellulose in the bagasse biomass and increased glucose yield ranges from 30 to 40%.

As shown in Fig 2., the sugar content increased steadily up to the glucose-xylose concentration decreased at 200 °C can be caused by the formation of low molecular weight compounds, such as acetic acid, hydroxy methyl furfural (5-HMF), and furfural due to secondary degradation of depolymerized carbohydrates [31].

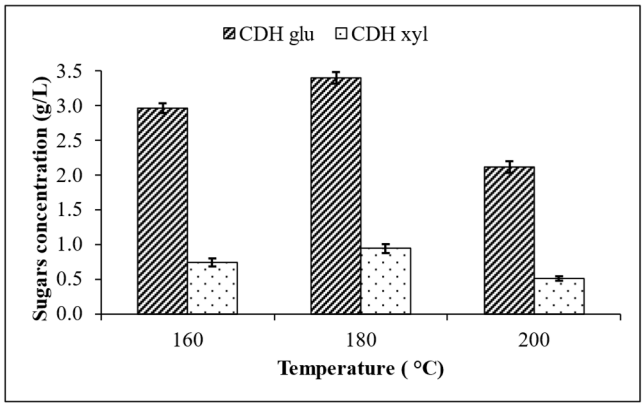

Fig. 2 Glucose-xylose concentration from hydrolysis of CPH powder under microwave irradiation in sulfuric acid medium at 160 – 200 °C. CDH glu: glucose, CDH xyl: xylose.

B. Oxidative-fermentative

Fermentation and oxidation play a major role in the accumulation of energy for microorganism activity and other biological processes. This test was obtained to inspect the ability of yeast to ferment xylose. The result is indicated by a change in the color of the indicator from blue to yellow (Fig. 3). These results showed that *S. cerevisiae* Y003 isolate could ferment pentose sugar (C5).

The color change of the media occurs because the OF medium contains bromothymol blue (BTB) as an indicator which will change color if there is a decrease in the pH of the media (Fig. 3). The pH drop indicates the presence of weak acid production during cell growth and ethanol fermentation [43], [44]. Pyruvate is converted into various mixed acids depending on the fermentation type, which is produced during the anaerobic fermentation process. The pyruvate produced from the glycolysis process will be converted to acetaldehyde by pyruvate decarboxylase. Alcohol dehydrogenase (ADH)

catalyzes the conversion of acetaldehyde to ethanol by oxidizing NADH to NAD+ and releasing CO_2, a by-product of the metabolic process. Another study reported that the amount of weak acid produced from yeast metabolism would react with the BTB indicator and cause a color change in the media [45], [46].

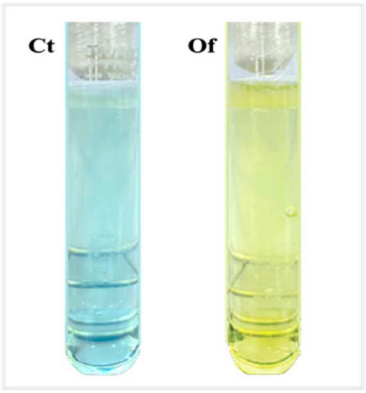

Fig. 3 Oxidative-fermentative test inoculated with *Saccharomyces cerevisiae* Y003. Ct: control, Of: medium inoculated with yeast isolate.

C. Stress Tolerance Assay

Evaluation of yeast cell viability against ethanol stress was carried out using the spot assay method by growing selected yeast isolates into YPD media (2% glucose) containing various concentrations of pH and ethanol with variations in incubation temperature (Fig. 4 and Fig. 5). As found in this study, yeast *S. cerevisiae* Y003 isolate was tolerant to high-temperature stress and exhibited growth following both high ethanol stress and pH stress conditions. *S. cerevisiae* Y003 grew well on spot media with up to 12% ethanol concentration at 40 °C incubation temperature and slightly decreased in media containing 14% ethanol (Fig. 4). Yeast Y003 isolate is resistant to pH stress (Fig. 5) and could grow in media with a pH stress range between pH 3 and pH 5 at all temperatures stress conditions. Isolate *S. cerevisiae* Y003 can be grown optimally at pH 5 and 30 °C incubation temperature. According to Ulya *et al.* [47] *P. kudriavzevii* 1P4 isolate exhibited a highly resistant phenotype to 10% ethanol.

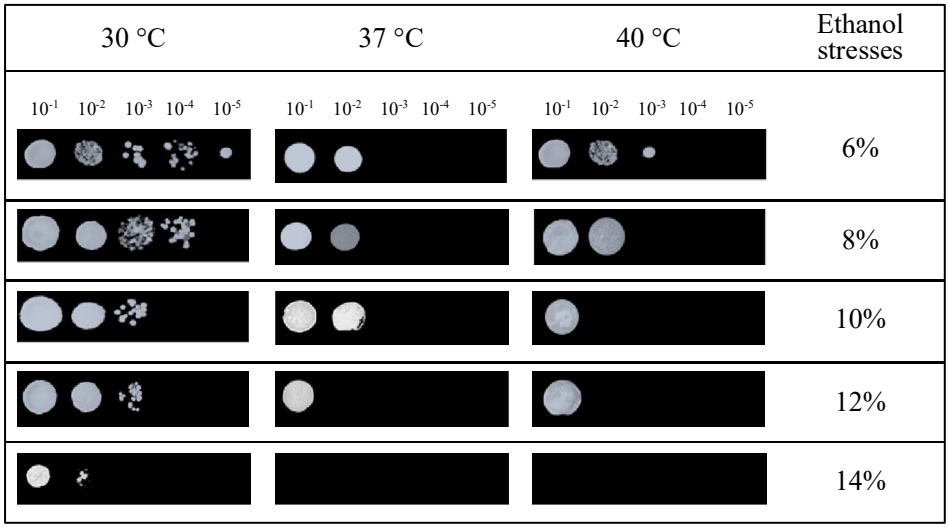

Fig. 4 Ethanol and temperature stresses effect on the cell growth of *S. cerevisiae* Y003 using spot assay method. The yeast Y003 isolate with preculture starting OD_{600} = 0.5 growth in YPD plate medium with ethanol stresses conditions (6%, 8%, 10%, 12%, 14%) and high temperature stresses (30 °C, 37 °C, 40 °C) with YPD plate was incubated for three days.

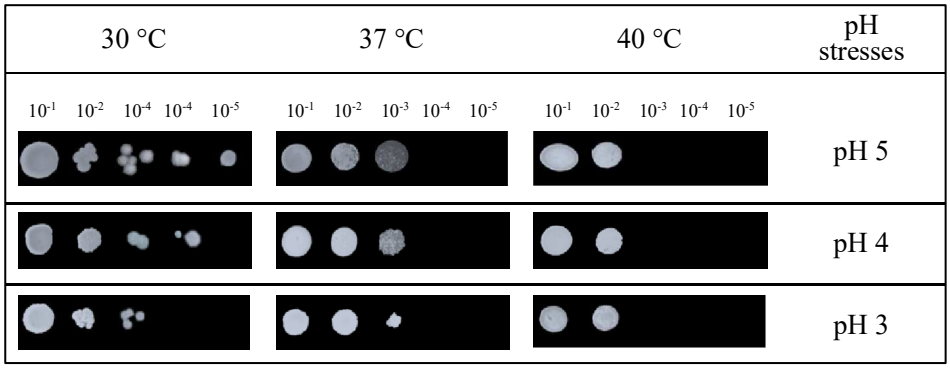

Fig. 5 pH and temperature stresses effect on the cell growth of *S. cerevisiae* Y003 using spot assay method. The yeast Y003 isolate with preculture starting OD_{600} = 0.5 growth in YPD plate medium with temperature stresses (30 °C, 37 °C, 40 °C) and pH stresses conditions (pH 3, pH4, pH5). YPD plate was incubated for three days.

Another study obtained mutant *P. kudriavzevii* R-T1, R-T2, and T-T2 exhibited higher survival rates than the wild-type strains under high ethanol stress (15%) [38], indicating that ethanol tolerance mechanism was in the different pathway from temperature and sugar tolerance mechanism. However, high ethanol concentrations in the medium can reduce cell vitality and lead to cell death in yeast [22], [23].

These results indicate that the stress tolerance mechanism in of *S. cerevisiae* Y003 essential against high temperature and pH stresses conditions but does not protect against high ethanol stress [38]. Based on this analysis yeast Y003, which showed characteristics of resistance to pH and high ethanol tolerance was used as a novel stress tolerance isolate in CPH biomass fermentation process.

D. Fermentation by S. cerevisiae

Fermentation activity and ethanol productivity of *S. cerevisiae* Y003 were assessed by the ability of isolate to ferment sugar monomers (glucose and xylose). Based on the measurements of glucose – xylose concentrations, yeast Y003 was able to spend almost all of the sugar content in medium, especially glucose (Fig. 6a-c). Glucose and xylose utilization was very efficient in the three fermentation conditions, with values ranging from 55.25% to100% (Tabel 2).

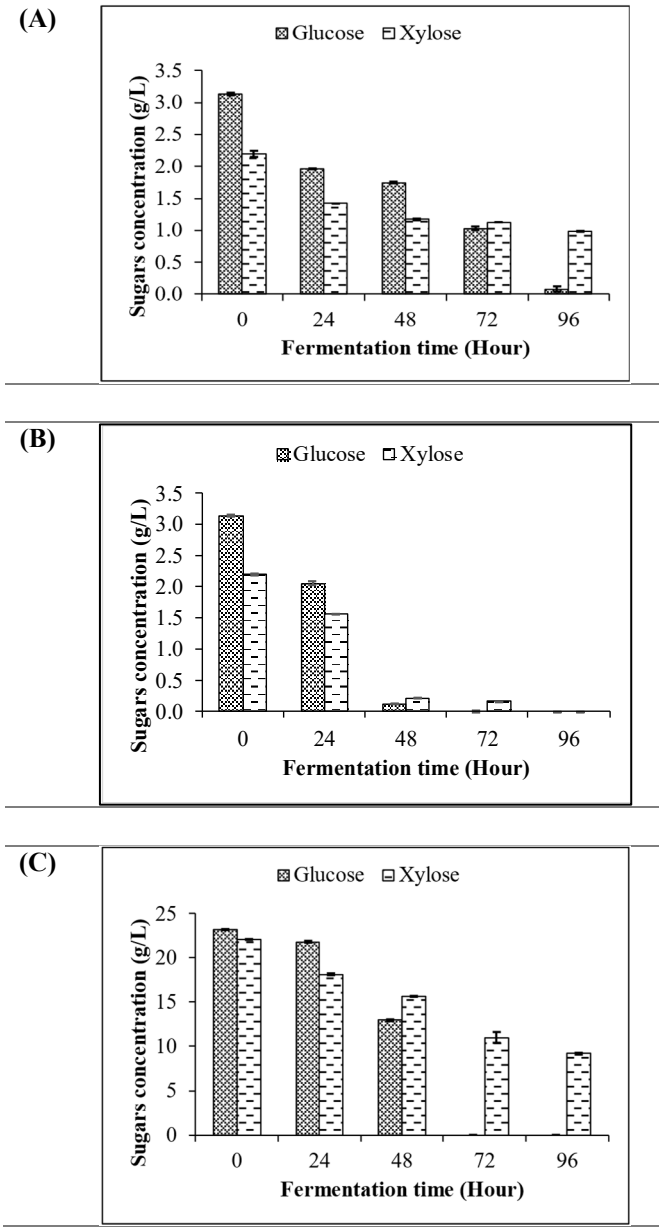

Fig. 6 Glucose and xylose concentrations during fermentation process by *S. cerevisiae* Y003 isolate with variations of fermentations substrate. A: substrate E1, B: substrate E2, C: substrate E3.

Sugars concentration decreased along with fermentation time. Significantly, the reduction in glucose concentration of each fermentation substrate began within 24 h of observation (Fig. 6a-c). *S. cerevisiae* Y003 reduce glucose and xylose concentration to 0.08 g/L and 0.98 g/L, respectively, with initial glucose and xylose were 3.13 g/L and 2.19 g/L (Table 2). On the other hand, yeast Y003 completely consumes glucose and xylose in E2 substrate at the end of the fermentation process (Fig. 6a). These results indicate that peptone and yeast extract influence high sugar consumption even at the same initial glucose and xylose concentration in the treated substrate. Addition of initial sugar concentration to E3 substrate obtained the opposite result due to use of xylose substrate was less effective than the E2 substrate.

The utilization on the E2 substrate was very efficient, with both glucose and xylose value of 100% after 96 h fermentation, and was the highest efficient value compared to other substrates (Table 2).

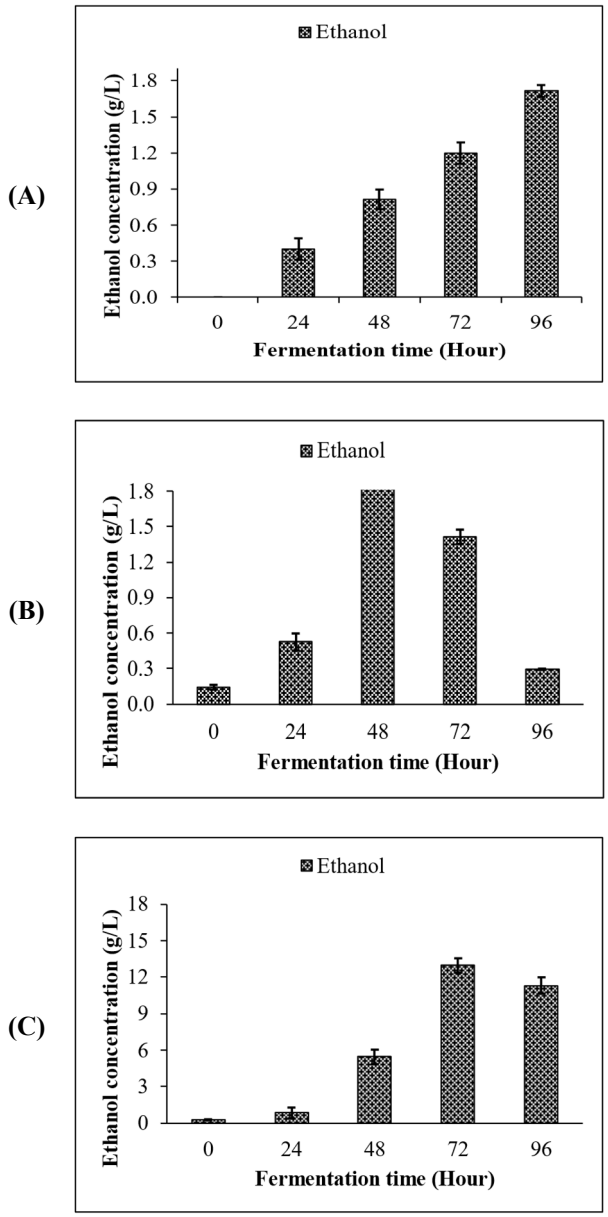

Fig. 7 Concentrations of ethanol produced during fermentation process by *S. cerevisiae* Y003 isolate with variations of fermentations substrate. A: substrate E1, B: substrate E2, C: substrate E3.

Increasing the first sugar concentration of the E3 substrate showed different results than the E2 substrate measurements, although both substrates were given the addition of yeast extract and peptone (Fig. 6c). Xylose consumption in E3 substrate was lower than E2 substrate, the utilization efficiency of xylose was only 58.37%. The high initial glucose concentrations implied that the utilization of xylose by yeast is not simultaneous. Sandoval *et al.* [48] reported that carbon catabolite repression (CCR) occurred in fermentation of mixed glucose-xylose substrates to avoid simultaneous use of both carbon sources. This study revealed that the best treatment condition was the variation of E2 substrate due to the simultaneous use of glucose and xylose. Adding peptone and yeast extract to the medium as a nitrogen source is thought to increase yeast cell growth and accelerate the fermentation process of glucose substrates.

The highest ethanol production of *S. cerevisiae* Y003 isolate was the E3 substrate of 12.98 g/L at 72 h observation time (Fig. 7a-c). The same initial sugar concentration on substrate E1 and E2 produces different concentrations of ethanol (Fig. 7a-b). The highest ethanol production on substrate E1 was 1.72 g/L at 96 hours of observation (Fig. 7a). The maximum ethanol concentration of E2 substrate after 48 h was 1.85 g/L, slightly higher than the ethanol production of E1 substrate at the same time, 0.82 g/L (Fig 7b). Ethanol production is related to sugar consumption because sugar substrates are not only used for ethanol production but are also required for the production of yeast cell biomass and other secondary metabolites such as glycerol, acetate, succinate and malate [49].

The kinetic parameters of ethanol production yeast Y003 in different fermentation media are shown in Table 3. The ethanol concentration of Y003 isolate increased along with increasing of media composition, including initial sugars and nutrients. The highest concentration of ethanol produced by *S. cerevisiae* Y003 was found in substrate E3 was 5.46 g/L (Table 3) at 48 hours of fermentation time. In this study, *S. cerevisiae* Y003 isolate increases the ethanol production as glucose is depleted. Glucose is used in the production and maintenance of yeast cells, moreover, glucose plays a main role in producing metabolic products.

Furthermore, Table 3 showed the highest ethanol yield and fermentation efficiency in E2 substrate (0.37 g/g and 97.09%) as the highest ethanol productivity was found in E3 substrate (0.11 g/L/h). Ethanol yield from three substrate showed slightly different value from 0.33 g/g – 0.37 g/L. As found in this study, ethanol yield and fermentation efficiency were achieved in media with high initial glucose and xylose. The ethanol productivity of Y003 on E3 fermentation substrate was 0.124 g/L/h, which was higher than other substrates.

TABLE II
SUBSTRAT UTILIZATION OF *S. CEREVISIAE* Y003 IN VARIOUS OF FERMENTATION MEDIA

Fermentation Substrate	Sugar	S0 (g/L)	S (g/L)	ΔS (g/L)	ΔS/S (%)
E1	Glucose	3.13±0.02	0.08±0.16	3.05±0.15	97.44±0.16
	Xylose	2.19±0.04	0.98±0.28	1.21±0.26	55.25±0.28
E2	Glucose	3.13±0.02	0.00±0.00	3.13±0.00	100.00±0.00
	Xylose	2.19±0.01	0.00±0.00	2.19±0.01	100.00±0.00
E3	Glucose	23.17±0.06	0.00±0.12	23.17±0.09	100.00±0.11
	Xylose	22.10±0.06	9.20±0.12	12.90±0.09	58.37±0.12

S0: initial sugar level (g/L), S: final sugar level, ΔS: consumed sugar level (g/L), ΔS/S: substrate utilization efficiency (%).

TABLE III
KINETIC PARAMETERS OF ETHANOL PRODUCTION BY *S. CEREVISIAE* Y003 AT 48 HOURS OF FERMENTATION

Fermentation Substrate	Glucose-xylose Consumption (%)	P(g/L)	Yp/s(g/g)	Qp (g/L/h)	Ey (%)
E1	45.23	0.82	0.34	0.02	90.82
E2	93.59	1.85	0.37	0.04	97.09
E3	36.68	5.46	0.33	0.11	85.32

P: ethanol produced during fermentation (g/L), Yp/s: ethanol yield (g/g), Qp: ethanol productivity (g/L/h), Ey: fermentation efficiency (%).

IV. CONCLUSION

This study found that the pretreatment process showed a higher reduction of lignin levels in CPH samples ICS 60 compared to TSH 858. The highest sugar concentration was obtained from hydrolysis using microwave-assisted hydrolysis at 180 °C for 12 minutes reaction time. Yeast *S. cerevisiae* Y003 showed resistance to fermentation stress up to 14% ethanol stress and pH stress level pH 3, and temperature stress up to 40 °C. The highest glucose-xylose consumption by *S. cerevisiae* Y003 was observed under the treatment of E2 (93.59%), with ethanol production up to 1.85 g/L. Fermentation efficiency from some variations of substrate fermentation was in the range of 85 – 97%.

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