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The Potential of Cellulolytic Yeast *Pichia manshurica* UNJCC Y-123, *Saccharomyces cerevisiae* UNCC Y-84, and *Saccharomyces cerevisiae* UNJCC Y-83 to Produce Cellulase Enzyme by Using Substrate Skin Delignification of Cocoa (*Theobroma cocoa*)

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Abstract

Exploring the possibility of using agricultural waste as a substrate for the synthesis of cellulase enzymes for fuel and renewable energy is the main goal of this study, which is in line with the notions of sustainable development and environmental stewardship. The product of delignification of cocoa peel can be used as a substrate for cellulase enzymes producing by yeast isolated from Balinese palm wine. Cellulase enzymes made from yeast from Balinese palm wine can be produced using the delignified cocoa peel as a substrate. This study aims to analyze the cellulase enzyme activity of yeast from Bali's palm wine on delignified cocoa bark substrates. The tests carried out were delignification of cocoa bark (solvent variations: HCl, H_2SO_4 and NaOH; concentrations: 1, 1.5 and 2 %; biomass 1:15 and 1:20 (w/v)); screening of cellulolytic yeast from Balinese palm wine; molecular identification and morphological characteristics of yeast; and cellulase enzyme activity (variation of yeast isolates and fermentation time of 48, 72 and 96 h). The results of delignification of cocoa shells showed that the use of 1.5 % NaOH solvent with cocoa shell powder biomass of 1:15 (9.59 ± 0.11) significantly differed from the value of reducing sugar content. Based on the screening results of 6 yeast isolates, 3 yeast isolates with the highest cellulolytic index values were selected, namely UNJCC Y-83 (0.29 ± 0.01 mm), UNJCC Y-123 (0.24 ± 0.01 mm) and UNJCC Y-84 (0.23 ± 0.01 mm). For testing cellulase enzyme activity. *S. cerevisiae* UNJCC Y-83 (4.11 ± 0.41 U/mL); *S. cerevisiae* UNJCC Y-84 (4.11 ± 0.33 U/mL) and *P. manshurica* UNJCC Y-123 (4.06 ± 0.12 U/mL) at 96 h of fermentation had significantly different cellulase enzyme activity. The results of identification of yeast rDNA in the D1/D2 region with NL1/NL4 primers obtained the identity of *Saccharomyces cerevisiae* UNJCC Y-83; *Saccharomyces cerevisiae* UNJCC Y-84 (99.66 % homology); and *Pichia manshurica* (100 % homology). *Theobroma cocoa* rind can be used as a yeast substrate to produce cellulase enzymes for fuel and renewable energy.

Keywords: Cellulase enzymes, Cellulolytic yeast, Cocoa skin, Delignification, Palm wine

Introduction

The skin cocoa contains lignocellulose which consists of 20.11 % lignin, 31.25 % cellulose and 48.64 % hemicellulose [1]. Cellulose in cocoa skin can be an alternative substrate for producing cellulase enzymes [2,3]. The delignification of cocoa skin must be carried out to produce cellulase enzymes [4-6]. Delignification is the process of overhauling the lignocellulosic structure so that it is more easily degraded into cellulose [7]. The delignification method can be carried out in various ways, physically and chemically [8]. Delignification is physically carried out by mechanical destruction such as grinding, while chemically, it is carried out by adding acidic and alkaline compounds [9]. Factors affecting delignification

are the chemical solvent compound type, concentration and amount of biomass [10,11]. Nazir *et al.* [8] reported that delignification of cocoa skin using 4 % NaOH with a biomass ratio of 1:25 (w/v) and 1.5 % H_2S0_4 with a biomass ratio of 1:16 (w/v) produced reducing sugars of 11.75 and 15. 59 %.

Cellulose will be hydrolyzed into glucose using cellulase enzymes after delignification [12,13]. The cellulase enzyme can hydrolyze the β-1,4 glucose bonds present in cellulose [14]. The cellulase enzyme group comprises an endoglucanase, exoglucanase and β-1,4 glucosidase [15]. Cellulase enzymes can be produced by various microorganisms, including yeast [13]. Yeasts that have the potential to produce cellulase enzymes are called cellulolytic yeasts [16]. Cellulolytic yeasts can hydrolyze cellulose in the environment and convert it into glucose monomers [17]. Several types of yeast known to have potential as cellulolytic yeasts are *Saccharomyces cerevisiae, Kluyveromyces marxianus, Trichocporon sporotrichoides, Candida sake, Mrakia blollopis, Mrakia gelida, Mrakia robertii* and *Wickermanomyces anomalus* [16,18].

The productivity of cellulase enzymes produced by yeast can be influenced by environmental factors [19]. Factors that affect cellulase enzyme activity are the type of inoculum, pH, substrate, temperature and incubation time [20]. Incubation time will affect yeast growth in the exponential phase of testing cellulase enzyme activity [21,22]. Thongekkaew *et al.* [23] reported that *Candida easanensis* using corn husk substrate at 96 h of fermentation time could produce cellulase enzyme activity of 0.089 U/mL.

Yeast can be isolated from fermented foods and beverages, such as palm wine from Bali [24]. Various types of yeast isolated from palm wine are *Saccharomyces cerevisiae* and *Candida tropicalis* [25,26] and the yeast *Saccharomyces* sp. isolated from wine from Nigeria [27]. Yeasts isolated from Burkina Faso wine belong to the genera *Arthroascus, Issatchenkia, Candida, Trichosporon, Hanseniaspora, Kodamaea, Schizosaccharomyces, Trigonopsis* and *Galactomyces* [28,29]. This study aims to screening of cellulolytic yeast from Balinese palm wine; molecular identification and morphological characteristics of yeast; and cellulase enzyme activity (variation of yeast isolates and fermentation time of 48, 72 and 96 h).

Materials and methods

Balinese palm wine yeast

A total of 6 yeast isolates from the Jakarta State University Culture Collection (UNJCC) collection from Balinese palm wine. The isolates used were yeast isolates with the code UNJCC Y-23, UNJCC Y-83, UNJCC Y-84, UNJCC Y-85, UNJCC Y-122 and UNJCC Y-123. The yeast *Saccharomyces cerevisiae* UNJCC Y-87 was used as a comparator isolate in testing cellulase enzyme activity. Cocoa skin samples were taken from the Sentul cocoa plantation, West Java.

Preparation of cocoa skin substrate

Cocoa skin sample preparation was carried out based on the method of Azelee *et al.* [30] with modifications. The 4,000 g of cocoa peel samples were washed using running water, then the samples were dried in the open air for 5 days and then dried in an oven at 60 °C for 24 h. The reduction in the weight of the cocoa skin after drying was calculated. Samples were crushed to a size of 100 mesh and used for further research.

Delignification of cocoa skin using acids and bases

Delignification was done using a method based on Sucihati *et al*. [1] with modifications. The delignification process was carried out in 3 stages: (1) Preparation of cocoa peel powder sample 1 g of cocoa peel powder dissolved in 15 and 20 mL of each solvent in 100 mL Erlenmeyer. (2) dissolving in types of acids and bases with different variations in the type and concentration of solvents used acid (HCl and H_2SO_4) and alkaline (NaOH) solvents, concentrations (1, 1.5 and 2 %) and biomass (1:15 and 1:20); (3) heating the cocoa skin powder sample has been drying oven for 2 min at 150 ℃ [31]. After the heating process, the filtrate and residue are separated. The filtrate is used to measure the concentration of reducing sugars. The residue of cocoa peel powder from delignification, with the highest concentration of reducing sugars, is rinsed and filtered using distilled water until the pH is neutral. The cocoa shell powder was then dried in an oven at 60 ℃ for 48 h [32]. The cocoa peel powder will be a substrate for cellulase enzymes.

Analysis of reducing sugar content

The reducing sugar calculation was carried out using standardization based on [33,34] using the reducing sugar test method using 3,5-dinitro salicylic acid (DNS). One mL of the delignified filtrate was homogenized with 1 mL of DNS reagent, vortexed for 1 min, and then incubated at 100 ℃ for 5 min. The solution was cooled, and the absorbance was calculated with a 540 nm UV Spectrophotometer. Glucose is used as a standard. A total of 1 mL of glucose with a concentration of 0, 0.5, 1, 1.5, 2 and 2.5 mg/mL. One mL of 3,5-Dinitrosalicylate (DNS) reagent was added 1 mL into each solution, homogenized using a vortex and then incubated at 100 ℃ for 5 min. Then the solution is cooled with running water. The solution is calculated absorbance wavelength of 540 nm.

Cellulase enzyme activity assay

Cellulase enzyme activity assay was carried out based on 3 stages: Preparation of yeast suspension, preparation of cellulase enzyme crude extract and yeast activity testing in producing cellulase enzyme. A total of 3 yeast isolates were used to test UNJCC Y-83, UNJCC Y-123 and *S. cerevisiae* UNJCC Y-84 as positive controls. The preparation of yeast cell suspension was determined using a spectrophotometer based on an Optical Density (OD) value of 1.0 at a wavelength of 600 nm (10^6 cfu/mL) [35,36]. The crude extract of the cellulase enzyme was made based on Sarawan *et al*. [15]. Fermentation was carried out using the Submerged fermentation (SmF) method with fermentation media containing 25 mL of yeast peptone broth media with the composition: 1 % yeast extract, 2 % peptone and 2 % cocoa shell powder which had been delignified in a 100 mL Erlenmeyer. The yeast cell suspension (10^6 cfu/mL) was inoculated into the fermentation medium. Incubation was carried out at 30 ℃ 150 rpm with 48, 72 and 96 h incubation times. The third stage is centrifugation. After fermentation, centrifugation was carried out at 3,000 rpm at 4 ℃ for 10 min. This crude enzyme extract is used to test cellulase enzyme activity.

Cellulase enzyme activity testing was carried out based on Souza *et al*. [13]. The cellulase enzyme activity test was carried out in 4 stages, namely mixing the crude extract of the enzyme with CMC substrate, administering DNS reagent, measuring glucose absorbance with a spectrophotometer and constructing a standard glucose curve [13]. The first stage is mixing the crude extract of the enzyme with the CMC substrate. The 0.5 mL of sample was a crude enzyme solution with 0.5 mL of 1 % CMC substrate, dissolved in 0.1 M sodium acetate buffer pH 5. Incubation was performed at 50 ℃ for 30 min [37]. The next stage is the provision of DNS reagents. A total of 1 mL of 3,5-Dinitrosalicylate (DNS) reagent with the sample was then homogenized using a vortex. Incubate at 100 ℃ for 15 min, then cool with distilled water. As much as 8 mL of sterile distilled water was added to the sample using a pipette and then homogenized again using a vortex. The third stage is the measurement of glucose absorbance which is measured using a spectrophotometer at a wavelength of 540 nm [33]. Measurement of cellulase enzyme activity based on Sahoo and Shirnali [18]:

$$Cellulase\ Enzyme\ activity\ (U/mL) = \frac{[\ Glucose\ ]\ x\ Fp}{BM\ Glucose\ x\ t\ x\ V}$$

Notes:

[Glucose] = Glucose content resulting from the hydrolysis of cellulose (µg)

Fp = Dilution factor

BM Glucose = Molecular weight of glucose (Mr = 180)

t = Incubation period (min)

V = volume of cellulase crude enzyme used (mL)

Molecular identification and morphological characterization of yeast

The yeast cell DNA genome was isolated using the boiling method [38]. The yeast cultures were cultivated in YM agar for DNA isolation. Cells were collected from the logarithmic phase were put into 0.5 mL Ultrapure distillate water by centrifugation at 13,000 rpm for 5 min. The DNA extract supernatant was transferred to a sterile tube and stored at −20 ℃. DNA amplification was carried out in the ribosomal region of the D1/D2 LSU 26S rDNA gene. The 25 µL of PCR reagent mixture consisting of forward primer NL 1 (5'-GCA TAT CAA TAA GCG GAG GAAAAG-3') and reverse primer NL 4 (5'-GGT CCG TGT TTC AAG ACG G-3') each 0.5 µL each with a concentration of 10 pmol; 12.5 µL GoTaq Green Master Mix (Promega); 10.5 µL NFW; and 1 µL DNA template [39]. PCR conditions: Pre-denaturation at 95 ℃ for 2 min, denaturation at 95 ℃ for 30 s, annealing at 60 ℃ for 30 s, extension at 72 ℃ for 1 min, final elongation at 72 ℃ for 10 min and cooling at 4 ℃ for 10 min. The reaction was carried out for 40

cycles [38]. Electrophoretic visualization was analyzed using 1 % agarose gel, Tris Acetate EDTA (TAE) buffer and 2 µL red gel. The PCR product was put into the agarose gel wells as much as 2 µL. A total of 2 µL of 100 bp DNA marker was used to determine the size of the band formed. Agarose gel was electrified at 110 volts for 15 min. The DNA fragments were observed using a UV transilluminator. The size of the resulting DNA bands ranges from 500 - 600 bp. Analysis of rDNA Sequences: The base sequence of the yeast PCR products was determined using sequence analysis. Chromas Pro software was used to assemble the DNA sequences that were acquired from the primer pairs. Using the Basic Local Alignment Search Tool Algorithm (BLAST), the obtained DNA sequences were compared to the database of identified sequences on the GenBank website (www.ncbi.nlm.nih.gov) to determine the homologous sequences [40]. The MEGA 7 program was then used to create phylogenetic trees using the modified sequences. The trees were rebuilt using the neighbor-joining (NJ) technique, and the evolutionary distances were accurately estimated using bootstrap values [38].

Results and discussion

Cocoa skin as a substrate to produce cellulase enzyme

The results showed that the cocoa shells that had been dried in the open air for 5 days resulted in a weight reduction of 3,078 g, then dried in an oven at 60 ℃ for 48 h, resulting in a weight reduction of 3,186 g (**Figure 1A**). The reduction in the weight of the cocoa skin is due to the reduced water content contained therein due to the drying process [41]. The third stage is manufacturing cocoa skin powder (**Figure 1B**). In this process, the cocoa skin is crushed mechanically using a blender. The goal is to reduce the size and structure of lignocellulosic so that cellulose is more easily accessible for further processing [42,43]. The reduced size of the biomass can increase its active surface area. The greater the surface area of the biomass, the more optimal the delignification process will be. After crushing using a blender, the cocoa shell powder is filtered using a sieve to obtain a uniform particle size of cocoa shell powder [34].

The cocoa skin used comes from ripe cocoa pods from the Sentul cocoa plantations. The characteristics of ripe cocoa fruit are yellow and oval, and the surface of the fruit is smooth. Ripe cocoa fruit is generally 4 - 5 months old [44]. The result of making cocoa shell substrate is cocoa shell powder a substrate. The preparation of the cocoa shell substrate was carried out in 3 stages: Washing the cocoa shell's surface, drying and making the cocoa shell powder [11]. The first step is washing the surface of the cocoa skin with running water. This process aims to clean the chocolate skin of microorganisms on the surface [43]. The second step is drying the cocoa skin in the open air. The goal is to avoid decay and reduce the water content in it. The third stage is oven drying at 60 ℃, which aims to reduce the remaining water content in the cocoa skin resulting from previous drying [41].

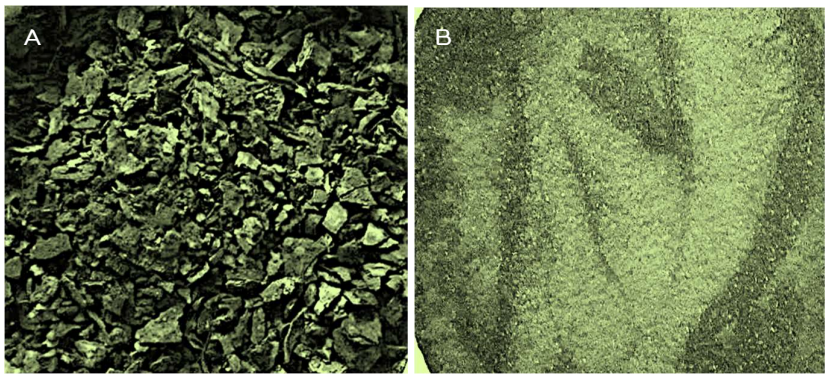

Figure 1 A) Cocoa shells that have been dried in the open air for 5 days and in the oven for 24 h and B) Cocoa skin powder that has been blended and filtered.

Delignification cocoa skin

In this study, cocoa shell powder was delignified using 3 different types of solvents, namely HCl, H_2SO_4 and NaOH, with a concentration of 1, 1.5 and 2 % and biomass of 1:15 and 1:20. The use of acids and bases is used as a solvent because they can break down the glycosidic bonds in the lignocellulosic cell walls and result in changes to the lignin structure, reduction of lignin and hemicellulose complexes and decrystallization of cellulose [4]. In the delignification process, there are 2 stages: Determining the standard glucose curve and the value of delignification reducing sugars. The statistical analysis results

using 3-way ANOVA show that the type of solvent, concentration, biomass and the interaction between the 3 has a Sig value. 0.00 < α (0.05) (**Table 1**), so the Duncan Multiple Range Test (DMRT) was carried out to determine significant differences in the interactions of the 3 treatments. The Duncan Multiple Range Test (DMRT) results showed that the interaction between the NaOH 1.5 % biomass 1:15 solvent was significantly different from the reducing sugar value, which was 9.59 ± 0.11. The treatment significantly differs more than α (0.05) on the 9 sub-sheets. It indicates that the treatment interaction on each sub-sheet is not significantly different from the reducing sugar value.

Table 1 Effect of type of solvent, concentration and biomass on the value of reducing sugar resulting from delignification of cocoa skin.

Type solvent	Concentration	Biomass (b/v)	
		1:15	**1:20**
NaOH	1 %	5.96 ± 0.32^{g}	6.02 ± 0.27^{g}
	1.5 %	**9.59 ± 0.11^{i}**	5.40 ± 0.60^{g}
	2 %	5.57 ± 0.27^{g}	6.98 ± 0.13^{h}
HCl	1 %	3.32 ± 0.23^{f}	2.60 ± 0.17^{f}
	1.5 %	0.53 ± 0.43^{abc}	1.06 ± 0.31^{bcd}
	2 %	1.70 ± 0.46^{de}	0.93 ± 0.12^{abcd}
H_2SO_4	1 %	0.40 ± 0.11^{ab}	2.48 ± 0.10^{ef}
	1.5 %	0.31 ± 0.10^{ab}	0.05 ± 0.05^{a}
	2 %	1.34 ± 0.34^{cd}	0.92 ± 0.10^{abcd}

The results showed that delignified cocoa shell powder using NaOH 1.5 % biomass 1:15 could be an optimal substrate because it produced the highest reducing sugar value. This is because basic compounds can break down ester bonds between lignin and hemicellulose, causing an increase in the porosity and surface area of the material, thereby increasing the availability of cellulose [45,50]. The NaOH molecule will dissolve the hemicellulose in the cocoa skin powder and break the intermolecular hydrogen bonds of the cellulose [11]. Cellulose will be unbound so that microorganisms easily hydrolyze it into glucose [46]. The value of reducing sugar produced in delignification using NaOH was higher than delignification using HCl and H_2SO_4. Kucharsk *et al.* [32] reported that using alkaline solvents resulted in smaller crystal sizes and lower crystallinity indexes than acidic solvents, so the lignin structure would be degraded. This could be due to alkaline solvents' higher ability than acidic solvents to hydrolyze cellulose [47].

The optimal concentration of NaOH in this study is 1.5 %. This is because an increase in concentration will reduce the glucose produced and will be further degraded into furfural compounds [48]. In contrast, a decrease in concentration can make cellulose difficult to hydrolyze. After all, lignin still protects it, so the reducing sugar produced is not maximal [46]. The optimal cocoa powder biomass in 1.5 % NaOH is a ratio of 1:15 (w/v). Cruz *et al*. [49] reported that a high amount of biomass will increase the viscosity, decrease cellulose's crystallinity, and increase hydrolysis's kinetic rate. In the delignification process, heating is carried out using an oven for 2 min to degrade the lignin polymer in the chocolate shell so that it dissolves with the solvent. Heating will cause more cellulose to be degraded because the lignin will dissolve [7]. Heating for a long time will reduce the formation of unwanted secondary products [34]. After being heated, the cocoa peel powder, dissolved with acid and base solvents, is filtered using filter paper. This aims to separate the delignified filtrate and residue. The delignified residue was then dried in an oven at 60 ℃ for 48 h. The aim is to dry the delignified chocolate powder as a substrate for cellulase enzymes.

The filtered delignified cocoa skin filtrate was measured for glucose absorbance to determine the value of reducing sugar. The cocoa skin filtrate was treated with 3,5-dinitro salicylic acid (DNS) reagent. This reagent has an aromatic compound that will react with reducing sugars to form 3-amino-5-nitrosalicylic acid. The compound can strongly absorb electromagnetic wave radiation at a wavelength of 540 nm. The more reducing components present in the sample, the more 3-amino-5-nitrosalicylic acid molecules will be formed, resulting in higher absorption. The reaction with DNS is redox on the aldehyde

group of sugar and is oxidized to a carboxyl group. DNS as an oxidizing agent, will be reduced to form 3-amino-5-nitrosalicylic acid. The reaction takes place in an alkaline environment. If there is reducing sugar in the sample, the solution will change from yellow to reddish-orange [34].

The screening of cellulolytic yeast isolates from Balinese palm wine

Screening of cellulolytic yeast from Balinese palm wine was carried out based on the method of Carrasco *et al*. [16] used the well method with agar diffusion. The media contains carboxymethylcellulose (CMC) as a carbon source which is water-soluble cellulose, yeast extract and agar. The agar diffusion method was used because yeast can degrade cellulose quickly in solid media [49]. Screening results showed 6 yeast isolates, UNJCC Y-83, UNJCC Y-123, UNJCC Y-84, UNJCC Y-122, UNJCC Y-23 and UNJCC Y-85, were able to produce cellulase enzymes (**Table 2**). A clear zone around the colony indicated this after dripping with congo red solution (**Figure 2**). The formation of a clear zone indicates that the yeast isolate can hydrolyze cellulose [18]. The descriptive statistical analysis results showed that the yeast isolate UNJCC Y-83 was significantly different from the cellulolytic index value of 0.29 ± 0.01. Yeast isolates UNJCC Y-123 and UNJCC Y-84 were not significantly different from cellulolytic index values, 0.24 ± 0.01 and 0.23 ± 0.01 mm. Yeast isolates UNJCC Y-122, UNJCC Y-23 and UNJCC Y-85 were not significantly different from cellulolytic index values, namely 0.12 ± 0.02, 0.11 ± 0.01 and 0.09 ± 0.03 mm (**Table 2**). Isolates capable of producing cellulase enzymes were marked by the clear zones that formed after being dripped with congo red. This is because congo red can bind to polysaccharides that have 1,4 β-glucoside bonds. The CMC substrate hydrolyzed to glucose by the cellulase enzyme (endoglucanase) produced by yeast cannot bind to congo red, causing a clear zone to form [24,51]. Different types of yeast produce different clear zones due to differences in their ability to degrade cellulose on CMC substrates.

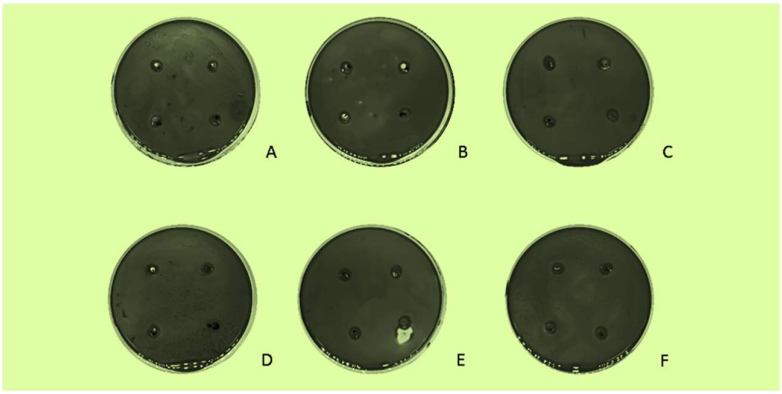

Figure 2 Screening of cellulase enzyme-producing yeast isolates on 1 % CMC media incubation 72 h after dripping with 0.1 % congo red A) UNJCC yeast isolates Y-83, B) UNJCC yeast isolates Y-123, C) UNJCC yeast isolates Y-84, D) yeast isolate UNJCC Y-122, E) yeast isolate UNJCC Y-23 and F) yeast isolate UNJCC Y-85.

Table 2 Cellulolytic index (IS) values of a yeast isolate from Balinese palm wine on CMC media incubated at 30 °C for 72 h.

Isolate number	Cellulolytic index value (mm)
	mean ± SE
UNJCC Y-83	**0.29 ±0.01**
UNJCC Y-123	**0.24 ± 0.01**
UNJCC Y-84	**0.23 ±0.01**
UNJCC Y-122	0.12 ± 0.02
UNJCC Y-23	0.11 ± 0.01

Identification yeast based on morphological and molecular characteristics

Identification was carried out molecularly on 3 yeast isolates with the highest cellulolytic index values: UNJCC Y-83, UNJCC Y-123 and UNJCC Y-84. Molecular identification was carried out on the D1/D1 region of ribosomal DNA. The D1/D2 area can be used as a barcode to identify yeast species by comparing the base pair divergence of yeast strains with DNA association values in existing databases [52]. The type of yeast isolate is determined by looking at the similarity to the BLAST results [53].

PCR amplification of yeast isolate from Balinese palm wine was performed on the D1/D2 ribosomal DNA using NL1/NL4 primers. The amplification results showed that the yeast isolate UNJCC Y-83 produced 592 bp DNA bands, the yeast isolate UNJCC Y-123 produced 560 bp DNA bands and the yeast isolate UNJCC Y-84 produced 591 bp DNA bands. Goldhawke *et al.* [54] reported that PCR amplification in the DNA ribosomal region D1/D2 using NL1/NL4 primers would produce DNA bands of 500 - 700 bp in size. Sumerta and Kanti [55] reported that yeast isolates amplified using the same primer produced DNA bands of 600 bp in size. The nucleotide base sequences of a yeast isolate UNJCC Y-83, UNJCC Y-123 and UNJCC Y-84 were compared with those stored in the National Center for Biotechnology Information (NCBI) genebank. The Basic Local Alignment Search Tool (BLAST) program was conducted to identify closely related species. The BLAST results showed that the yeast isolates UNJCC Y-83 and UNJCC Y-84 had the closest homology to *S. cerevisiae* NRLL Y 12632 with an identity value of 99.66 %. In comparison, the yeast isolate UNJCC Y-123 had the closest homology to *P. manshurica* IFO 10726 with a value of 100 % identity (**Table 3**).

In the nucleotide alignment results of the yeast isolate UNJCC Y-83, there was a gap and difference of 1 out of 592 nucleotides (0 %). In contrast, the yeast isolates UNJCC Y-123 and UNJCC Y-84 did not have a gap in the nucleotide base sequence of *S. cerevisiae* NRLL Y 12632 and *P. manshurica* IFO 10726 (**Table 3**). If the yeast strain in the database has a difference of no more than 3 nucleotides in 500 - 600 nucleotides, it can be said to be the same species. Meanwhile, a difference of 6 or more nucleotides (1 %) indicates that the species differ [53].

Table 3 BLAST results of yeast isolates UNJCC Y-83, UNJCC Y-123 and UNJCC Y-84 based on analysis of the D1/D2 rDNA region sequences.

Isolate number	Takson identified from BLAST	*Max score*	*Query cover*	*E-value*	*Acession*	*Identity*
UNJCC Y-83	*Saccharomyces cerevisiae* NRRL Y 12632	1,083	95 %	0	NG_042623.1	99.66 %
UNJCC Y-123	*Pichia manshurica* IFO 10726	1,035	92 %	0	NG_055078.1	100 %
UNJCC Y-84	*Saccharomyces cerevisiae* NRRL Y 12632	1,083	95 %	0	NG_042623.1	99.66 %

The Neighbor-joining method was used to reconstruct the phylogenetic tree with a bootstrap value of 1,000 replications in an effort to determine the lowest evolutionary gap between each yeast species [56,55]. The findings revealed that the yeast UNJCC Y-123 was in a monophyletic clade with yeast P. manshurica IFO 10726 type strain with 100 % bootstrap value, while the yeast UNJCC Y-83 and UNCC Y-84 were in a monophyletic clade with yeast S. cerevisiae NRLL Y 12632 type strain with a bootstrap value of 92 % (**Figure 3**) [57,58]. The bootstrap results give phylogenetic trees confidence ranges and show that the measurement accuracy is greater than 70 % [57], supporting the idea that the species belong to the same clade.

Aspergillus niger was used as an outgroup in reconstructing the phylogenetic tree based on sequence analysis of the D1/D2 regions [59]. Michu [60] reported that an outgroup is a species not included in the species group under study. Outgroups can be selected based on previous literature, research or spotted while aligning sequences. *S. cerevisiae* and *P. manshurica* are yeasts belonging to the order Saccharomycetales and the phylum Ascomycota. *Saccharomyces cerevisiae* comes from the genus *Saccharomyces*, while *Pichia manshurica* comes from *Pichia*. This yeast plays an important role in fermentation, and bread-making can be found in plants, fruits and soil [61]. *S. cerevisiae* and *P. manshurica* are known to produce cellulase enzymes [62]. These 2 types of yeast are commonly found in palm wine and can produce bioethanol which can live in aerobic and anaerobic conditions [63].

Morphological characterization of cellulolytic yeast from Balinese palm wine was carried out by observing the morphology macroscopically and microscopically (**Figure 4**). Macroscopic observations were made to observe the morphology of the yeast *S. cerevisiae* UNJCC Y-83, *P. manshurica* UNJCC Y-123 and *S. cerevisiae* UNJCC Y-84 on YMA media for 48 h using a digital stereo microscope. Microscopic observations were made to see yeast cells using a light microscope (**Figure 4**).

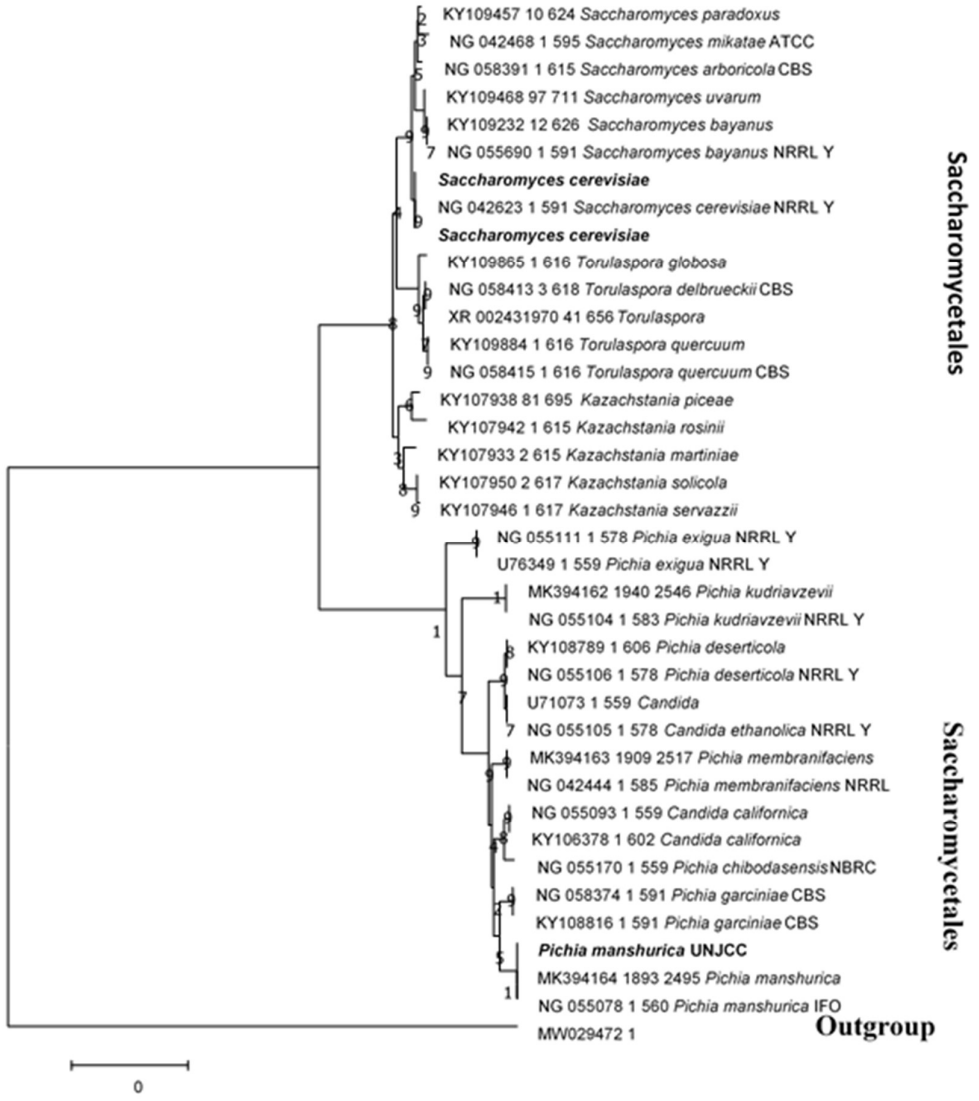

Figure 3 Yeast phylogenetic tree UNJCC Y-83, UNJCC Y-123 and UNJCC Y-84 with D1/D2 area analysis, neighbour-joining method with 1,000× bootstrap.

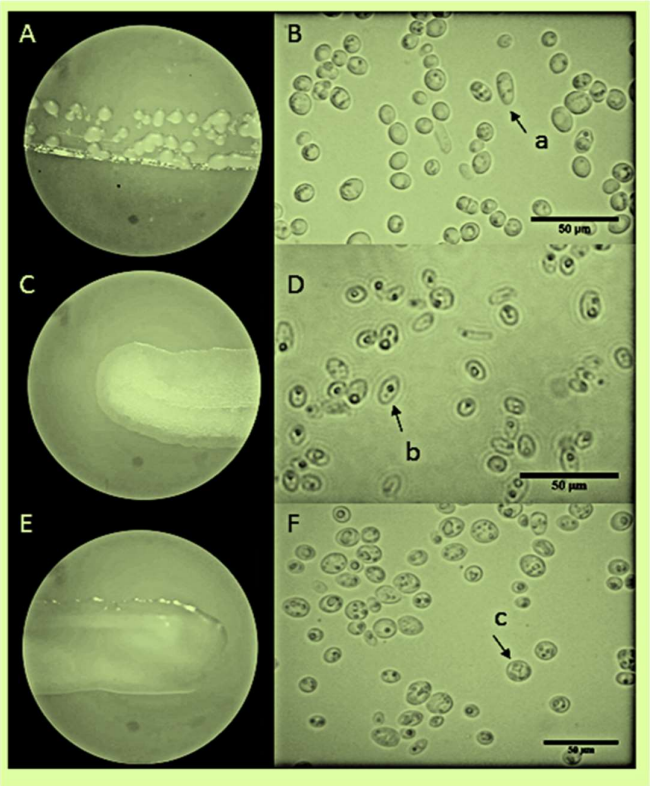

Figure 4 Macroscopic and microscopic morphology of cellulolytic yeast from Balinese palm wine on YMA medium at 30 °C incubation for 48 h with 400× magnification. A) macroscopic *S. cerevisiae* UNJCC Y-83, B) yeast cells of *S. cerevisiae* UNJCC Y-83, C) macroscopic *P. manshurica* UNJCC Y-123, D) yeast cells of *P. manshurica* UNJCC Y-123, E) macroscopic *S. cerevisiae* UNJCC Y-84 and F) yeast cells *S. cerevisiae* UNJCC Y-84; a) monopolar budding, b) oval-shaped yeast cells and c) round yeast cells.

The results of macroscopic observations showed that *S. cerevisiae* UNJCC Y-83 has the characteristics of white colour, circular shape, mucoid texture, smooth surface, flat edges and convex elevation. *P. manshurica* UNJCC Y-123 has characteristics of white colour, circular shape, butyrous texture, rough surface, flat edge and flat elevation. The macroscopic characteristics of *S. cerevisiae* UNJCC Y-84 are white, circular in shape, mucoid texture, smooth surface, flat edges and raised elevation (**Table 4**). The microscopic morphological characteristics of *P. manshurica* UNJCC Y-123 are round and oval cell shapes, monopolar budding and no hyphae. *S. cerevisiae* UNJCC Y-83 and *S. cerevisiae* UNJCC Y-84 have the characteristics of round-shaped cells, monopolar budding and no hyphae (**Table 5**).

Table 4 Macroscopic observation of yeast from Balinese palm wine on YMA medium, incubated at 30 °C for 48 h.

Khamir isolate	Isolate number	Colour	Form	Texture	Surface	Side	Elevation
Saccharomyces cerevisiae	UNJCC Y-83	White	circular	mucoid	smooth	flat	convex
Pichia manshurica	UNJCC Y-123	White	circular	butyrous	rough	flat	flat
Saccharomyces Cerevisiae	UNJCC Y-84	White	circular	mucoid	smooth	flat	raised

Table 5 Microscopic observation of yeast from Balinese palm wine on YMA medium, incubated at 30 °C for 48 h.

Yeast isolate	Isolate number	Cell shape	Budding	Hyphae
Saccharomyces cerevisiae	UNJCC Y-83	round	monopolar	none
Pichia manshurica	UNJCC Y-123	round, oval	monopolar	none
Saccharomyces cerevisiae	UNJCC Y-84	round	monopolar	none

The ability of yeast from Balinese palm wine to produce cellulase enzymes

Cellulase enzyme activity was tested on 3 yeast isolates from Balinese palm wine with significant differences in cellulolytic index values, *S. cerevisiae* UNJCC Y-83, *P. manshurica* UNJCC Y-123 and *S. cerevisiae* UNJCC Y-84 (**Table 6**). Another isolate is *Saccharomyces cerevisiae* UNJCC Y-87 as a comparison yeast isolate. Using comparator isolates were used to compare the potential of yeast from different isolation origins, namely Brem Lombok, in producing cellulase enzymes. *S. cerevisiae* is a yeast used by industry to produce cellulase enzymes [64].

The yeast fermentation process used the Submerged fermentation (SmF) method under aerobic conditions [65]. The SmF method was chosen because of the availability of nutrients and oxygen that easily dissolve in the liquid medium and are evenly distributed throughout the container so that heat and mass transfer takes place efficiently. Environmental conditions such as temperature and pH will stabilize during fermentation [66]. The 2-way ANOVA analysis showed that the interaction between yeast isolates, fermentation time and the interaction between the 2 on the enzyme activity value had a Sig value. $0.00 < \alpha$ (0.05) (**Table 6**), so the Duncan Multiple Range Test (DMRT) was carried out to find out the real difference. The results of the Duncan Multiple Range Test (DMRT) advanced tests showed that the interaction between the yeast isolates *S. cerevisiae* UNJCC Y-83, *S. cerevisiae* UNJCC Y-84 and *P. manshurica* UNJCC Y-123, with a fermentation time of 96 h, was significantly different from the cellulase enzyme activity that is equal to 4.11 ± 0.41, 4.11 ± 0.33 and 4.06 ± 0.12 (**Table 6**). On sub sheets a, b and c, it has a significantly more than α (0.05). This indicates that the treatment interaction on each sub-sheet was not significantly different from the value of cellulase enzyme activity.

Table 6 Test results of cellulase enzyme activity of *S. cerevisiae* UNJCC Y-83, *P. manshurica* UNJCC Y-123 and *S. cerevisiae* UNJCC Y-84 with fermentation times of 48, 72 and 96 h incubation at 30 °C.

Yeast isolate	Isolate number	Cellulase enzyme activity (U/mL)		
		48 h	72 h	96 h
S. cerevisiae	UNJCC Y-83	1.74 ± 0.08[a]	1.77 ± 0.16[a]	**4.11 ± 0.41**[c]
P. manshurica	UNJCC Y-123	1.74 ± 0.07[a]	2.06 ± 0.28[a]	**4.06 ± 0.12**[c]
S. cerevisiae	UNJCC Y-84	1.53 ±0.12[a]	1.65 ± 0.03[a]	**4.11 ± 0.33**[c]

The fermentation medium includes cocoa skin powder, yeast extract and peptone. Cocoa skin is a carbon source because it consists of cellulose that can be hydrolyzed into glucose monomers [67]. The cocoa peel powder used results from optimal delignification using NaOH 1.5 % biomass 1:15, so its cellulose content will be higher. Yeast extract and peptone contained in the media function as a source of nitrogen. Rotary shaker agitation aims to provide aeration or oxygen exchange, mass heat transfer and good substrate mixing [68]. Centrifugation was carried out at 3,000 rpm at 4 ℃ for 10 min. The purpose of centrifugation is to separate insoluble products, such as yeast cells. The cellulase enzymes are extracellular (secreted by cells into the external environment), so they remain in the supernatant when the biomass is removed [69]. The supernatant was used to test the cellulase enzyme activity.

Cellulase enzyme activity was tested by mixing 1 % CMC substrate dissolved in acetate buffer pH 5.0 with crude enzyme extract from the sample. Acetate buffer pH 5 was used as a solvent to maintain the stability of the substrate pH [70]. The mixture was then incubated at 50 ℃, as Touijer *et al.* [37] reported

that yeast can hydrolyze cellulose substrates at high temperatures and optimally at 50 ℃. It is crucial to ensure enzyme stability for achieving high enzyme activity values [71].

The results showed that *S. cerevisiae* UNJCC Y-83, *S. cerevisiae* UNJCC Y-84, *P. manshurica* UNJCC Y-123 at 96 h of fermentation time, was significantly different from the value of cellulase enzyme activity, which was 4.11 ± 0.41, 4.11 ± 0.33 and 4.06 ± 0.12, respectively. Oh and Jin [72]; Ospina *et al*. [61] reported that *S. cerevisiae* and *P. manshurica* could produce cellulase enzymes. Amazee *et al.* [73] reported that S. *cerevisiae* isolated from palm wine produced cellulase enzyme activity values on pineapple peel and orange peel substrates of 0.330 mg/mL and 0.251 U/mL at 72 and 96 h of incubation. Qadir *et al*. [74] reported that the cellulase enzyme activity on bagasse substrate using co-culture of the yeast *S. cerevisiae* and *C. tropicalis* was optimum at 94 h of fermentation, which was 9.81 U/mL. Amadi *et al.* [70] reported that the peak production of enzymes for most microorganisms occurs during the exponential growth phase. Yeast will grow quickly in optimal media and provide high enzyme activity values. The production of enzymes depends on the chemical composition of the substrate, accessibility and physiochemical associations between its components.

The results of this study indicate that the cellulase enzyme activity of the 3 selected yeast isolates, *S. cerevisiae* UNJCC Y-83, *S. cerevisiae* UNJCC Y-84, and *P. manshurica* UNJCC Y-123, differed significantly at 96 h of fermentation time. These findings are important because they suggest that different yeast strains have varying abilities to produce cellulase enzymes, which could impact the efficiency of the enzymatic hydrolysis process. The differences in cellulase enzyme activity observed among the 3 yeast strains could be attributed to several factors, including genetic differences between the strains and variations in fermentation conditions. For example, it is possible that one strain has a higher expression level of cellulase genes than the others, leading to a greater production of enzymes. Additionally, differences in environmental conditions during fermentation, such as temperature, pH, and nutrient availability, could also impact the ability of the yeast to produce cellulase enzymes. These findings are consistent with previous research that has shown variations in cellulase enzyme activity among different yeast strains. For example, a study by Mbajiuka *et al.* [36] found that 2 different strains of *S. cerevisiae* had significantly different cellulase enzyme activity levels. Similarly, a study by Touijer *et al.* [37] found that several different strains of *P. manshurica* had varying abilities to produce cellulase enzymes. Overall, these results highlight the importance of selecting the appropriate yeast strain for cellulase enzyme production, as different strains can have significant differences in enzyme activity. This information could be useful for improving the efficiency and cost-effectiveness of enzymatic hydrolysis processes, which are used for the production of biofuels and other renewable energy sources.

Conclusions

The screening test results for 6 yeast isolates from Bali's palm wine: UNJCC Y-83, UNJCC Y-123, UNJCC Y-84, UNJCC Y-122, UNJCC Y-23 and UNJCC Y-85 have cellulolytic potential by producing cellulase enzymes. The results of rDNA sequence analysis in the D1/D2 region showed that the yeast isolates UNJCC Y-83 and UNJCC Y-84 had a homology level of 99.66 % as *Saccharomyces cerevisiae* with a bootstrap value of 92 %. UNJCC Y-123 yeast isolate has a homology level of 100 % as *Pichia manshurica* with a bootstrap value of 100 %. There is an influence of yeast isolate, fermentation time and interaction between the 2 factors on the value of cellulase enzyme activity, where the yeast *S. cerevisiae* UNJCC Y-83, *S. cerevisiae* UNJCC Y-84, and *P. manshurica* UNJCC Y-123 at 96 h of different fermentation time significantly to the value of cellulase enzyme activity, respectively 4.11 ± 0.41, 4.11 ± 0.33 and 4.08 ± 0.12 U/mL.

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