Isolation of thermo-tolerant and ethanol-tolerant yeast from local fermented foods and their potential as bioethanol producers

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Abstract. Bioethanol is a liquid chemical produced from sugar-, starch- or lignocellulosic-based biomass through fermentation by ethanol-producing microbes. Ethanol-producing yeast generally has limited tolerance to ethanol and has limitation to high temperatures above 40°C. High-temperature tolerant yeast is required because it potentially reduces the risk of contamination and also reduces the cost of the cooling process. This study aims to determine ethanol-producing yeasts that have tolerance to ethanol and high temperatures from local fermented food products. This study uses a descriptive method conducted in three stages. Isolation and selection of yeast were performed from 18 local fermented foods in Indonesia. Temperature and ethanol tolerance of selected yeast were performed by using a spot test method. The ethanol content was tested using Gas Chromatography (GC). The results exhibited that isolate F08b had the highest tolerance to ethanol and temperature. The isolate was able to grow up to a temperature of 50°C and a concentration of 18% ethanol. Meanwhile, isolate F10 was able to produce the highest ethanol concentration at 3.37% (v/v) in 48th-hour fermentation.

1. Introduction

Bioethanol is the metabolite produced from glucose-containing materials through fermentation by beneficial microorganisms such as bacteria and yeast. Conventional yeast which acts as a bioethanol producer has a limited tolerance to temperatures above 40°C and has a limited tolerance to the level of ethanol concentration above 20% [1]. As a result, yeast will unable to grow during the fermentation process as well as unable to produce bioethanol. The ethanol-producing yeast that has resistant to heat and high ethanol concentration is necessary for the development of industrial biofuel. The thermo-tolerant yeasts are able to survive at high temperatures of 40°C or above. The advantage of fermentation at high temperatures is not only to reduce the risk of contamination but also to reduce the cooling cost during ethanol production [2,3]. While yeast can be categorized into ethanol tolerant depend on their ability to survive in an environment that has an ethanol concentration of 10% or more [4].

In the previous studies, some thermo-tolerant and ethanol-tolerant yeast were obtained from local fruits such as papaya, banana, star apple, and cacao [5]. Some thermo-tolerant and ethanol-tolerant yeast were also obtained from local vegetables such as squash, green beans, carrot, kale, and celery [6]. However, there is limited information on thermo-tolerant yeast from Indonesian fermented foods. The potential of yeast as a producer of ethanol from local fermented food products in Indonesia has been investigated through the use of several substrates. In general, the yeast obtained from fermented foods
in Karimun Besar Island, Riau Archipelago Province are identified as Saccharomyces cerevisiae, Torulaspora delbrueckii, Torulaspora globose, Candida glabrata, Kodamaea ohmeri, Pichia kudriavzevii [7].

In this study, Indonesian local fermented foods were subjected as the focus of yeast isolation because it contains some nutrients such as sugars and micronutrients for yeast growth and there was limited thermo-tolerant yeast from Indonesian fermented foods. This research aimed to isolate and characterize thermotolerant yeast from the local fermented foods (from (Peuyeum, Balinese Brem, Solid Brem, Cassava Tape, Tauco, Soy Sauce, Balinese Arak, Mandai, Lempok, Gaplek, Shrimp Bekasam, Tempoyak, Fish Bekasam, Pede, Rusip, Growol, Tempe Gembus, Tempe Kacang, Tuak, Water of sticky rice). These properties are crucial for the development of the ethanol industry due to some benefits such as economic cooling cost and low risk of contamination during fermentation [3,8,9]. In order to explore new candidate of thermo-tolerant and ethanol-tolerant yeast isolates from Indonesian fermented foods, isolation, screening, and characterization is essential to be investigated.

2. Materials and methods

2.1. Materials

The local fermented foods (Peuyeum, Balinese Brem, Solid Brem, Cassava Tape, Tauco, Soy Sauce, Balinese Arak, Mandai, Lempok, Gaplek, Shrimp Bekasam, Tempoyak, Fish Bekasam, Pede, Rusip, Growol, Tempe Gembus, Tempe Kacang, Tuak, Water of sticky rice) were obtained from various regions in Indonesia. The media used in this study were Yeast extract Peptone Glucose Agar (YPGA) and Yeast extract Peptone Glucose Broth (YPGB). The D-glucose was purchase from Sigma, peptone (Kyokuto), yeast extract (Nacalai Tesque), chloramphenicol (Sigma), methylene blue (Sigma), other chemicals used in this study were of analytical grade.

2.2. Equipment

The equipment used in this study were autoclave (TOMMY), electric stove (Maspion), incubator (Binder), digital scale (Metler Denver AA 200), oven (Binder), laminar air flow (local), vortex, spectrophotometer (Unico UV-2100), colony counter brand (WTW BZG 30), micropipette (Gilson), shaker waterbath (Julabo SW22), refrigerator, cold centrifuge (Hettich Zentrifugen Mikro 22R), microscope (Olympus optical Co.Ltd), Gas Chromatography Flame Ionization Detector (Agilent Technologies), and standard glassware.

2.3. Yeast isolation

The Indonesian fermented foods were taken and weighed as much as five grams and then dissolved into an Erlenmeyer flask containing 45 ml of sterile distilled water and then homogenized. The solution then was diluted into 9 ml of YPG broth contains 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) D-glucose. The samples were incubated at 30°C for 48 hours. About 1 ml diluted sample was spread on YPG agar and incubated at 30°C for 24 hours. The colonies resembling yeast characteristics were chosen and inoculated on YPG agar with the addition of 0.05% (w/v) chloramphenicol, and incubated at 30°C for 24 hours [10] with slight modification.

2.4. Screening of thermo-tolerant yeast

The isolates were inoculated in a test tube containing 10 ml of YPG broth and incubated at 30°C with agitation 100 rpm for 18 hours. The cells were harvested by centrifugation at 5,000 rpm, 4°C for 5 minutes. The pellets were collected and then washed with sterile distilled water and repeated twice. The initial OD value was determined at the absorbance of 660nm and the culture volume and the addition of sterile distilled water were calculated. Dilutions of 10^0, 10^{-1}, 10^{-2}, 10^{-3} were carried out. Each dilution was inoculated using a 5µl micropipette according to a spotted test method [9,11] and incubated for 48 hours at temperatures of 37°C, 40°C, 45°C, 48°C, 50°C, and 52°C [9] with slight modification in temperature range and volume of culture for spotted test. For a spotting test, after pre-culture in YPG
medium, the initial OD$_{660}$ was adjusted to 1 with a fresh medium. One milliliter of the adjusted pre-culture was subjected to low-speed centrifugation, and the pellet of cells was washed with deionized water, suspended in deionized water at approximately $1 \times 10^7$ cells ml$^{-1}$, 10-fold sequentially diluted, and spotted onto YPG plates [6].

2.5. Screening of ethanol-tolerant yeast

The isolates were inoculated in a test tube containing 10 ml of YPG broth, and incubated at 30°C with agitation 100 rpm for 18 hours. The cells were harvested by centrifugation at 5,000 rpm, 4°C for 5 minutes. The pellets were taken and then washed with sterile distilled water and carried out twice. The initial OD value was determined at the absorbance of 660nm and the culture volume and the addition of sterile distilled water were calculated. Dilutions of $10^{-1}$, $10^{-2}$, $10^{-3}$ were carried out. Each dilution was inoculated using a 5µl micropipette according to a spotted test method [9,11] on YPG agar at different concentrations, namely 10%, 12%, 15%, 16%, 17%, and 18% and incubated at 30°C for 48 hours [9] with slight modification in ethanol concentration range and volume of culture for the spotted test.

2.6. Ethanol production of several yeast isolates

The ethanol production test was carried out by taking one loop of a single colony based on the final log phase of each isolate, then inoculated in YPG broth at 40°C overnight. The pre-culture then was inoculated in YP broth containing 10% glucose and incubated at 40°C. Ethanol concentration was observed at 12, 24, and 48 hours. The analysis of ethanol concentration was using Gas Chromatography with simultaneous Flame-Ionization (GC-FID) with slight modification [6,12].

3. Results and discussion

3.1. Thermo-tolerant yeast from local fermented foods in Indonesia

Thermo-tolerant yeast was carried out to test the level of resistance of yeast to a certain temperature level. The thermo-tolerant yeasts were tested by using the spotted test method [11]. The result of the thermo-tolerant yeasts was shown in Table 1.

| Yeast isolates | 37 | 40 | 45 | 48 | 50 | 52 |
|---------------|----|----|----|----|----|----|
| F01           | +++| +++| +++| +++| -  | -  |
| F02           | +++| +++| +++| +++| -  | -  |
| F08a          | +++| +++| +++| +++| +  | -  |
| F08b          | +++| +++| +++| +++| +  | -  |
| F08c          | +++| +++| +++| +++| -  | -  |
| F10           | +++| +++| +++|+++| -  | -  |
| F13           | +++|+++|+++|+++| -  | -  |

Notes:
All the yeast isolates were analyzed according to [6]. The yeast isolates with +++[+] very strong growth on YPG agar, indicated by the growth ability at dilution 10$^{-3}$; isolates with +++[+] strong growth, ability to grow at dilution 10$^{-2}$; isolates with ++[+] medium growth, ability to grow at dilution 10$^{-1}$, isolates with [+][+] less growth, ability to grow at 10$^{0}$, [-][-] isolates cannot grow.

In Table 1, all isolates, namely F01, F02, F08a, F08b, F08c, F10 and F13, exhibited good growth up to a temperature of 48°C. While at a temperature of 52°C all isolates could not grow on YPG agar media. According to the heat resistance test, the best results were obtained from isolates F08a and F08b which still showed their ability to grow at a temperature of 50°C even though their growth was weak. Caspeta and Nielsen state that computational analysis of the physical properties of proteins shows that the lethal
temperature for yeast is around 49°C [13]. The phenomenon occurred due to protein denaturation among most of the yeasts. The ability of yeast isolates to tolerate temperature depends on the cell membrane. According to Arora et al, thermotolerant microorganisms can produce different enzymes that function under extreme conditions, so that they are still able to metabolize and grow [14]. Several factors that can affect the growth of high temperature resistant yeasts are certain chemicals, osmotic dehydration, and low external pH, the composition of culture media, and growth phase [15]. Yeast strains that have resistance to high temperatures can survive at temperatures above 40°C [16]. Kuroda and Ueda stated that heat shock response (HSP) makes the molecular mechanism in cells more tolerant to the temperature. Specific transcription process can activate the chaperone protein gene (HSP) in response to high temperatures [17].

3.2. Ethanol-tolerant yeast from local fermented foods in Indonesia

Yeast tolerance test for ethanol was carried out to determine isolates that had high resistance to ethanol. The amount of ethanol in the given media was 10%, 12%, 15%, 16%, 17%, and 18%. The results of the ethanol-tolerant yeasts were shown in Table 2.

### Table 2. Growth of yeast isolates from local fermented foods under various ethanol concentrations.

| Yeast Isolates | Ethanol Concentration (%) |
|---------------|---------------------------|
|               | 10 | 12 | 15 | 16 | 17 | 18 |
| F01           | ++++ | ++++ | ++++ | ++++ | ++ | + |
| F02           | ++++ | ++++ | ++++ | ++++ | +  | -  |
| F08a          | ++++ | ++++ | ++++ | ++++ | ++ | -  |
| F08b          | ++++ | ++++ | ++++ | ++++ | ++++ | ++ |
| F08c          | ++++ | ++++ | ++++ | ++++ | +++ | -  |
| F10           | ++++ | ++++ | ++++ | ++++ | ++ | -  |
| F13           | ++++ | ++++ | ++++ | ++++ | ++ | -  |

Notes:

All the yeast isolates were analyzed according to [6]. The yeast isolates with [++++] very strong growth on YPG agar, indicated by the growth ability at dilution 10⁻³, isolates with [+++] strong growth, ability to grow at dilution 10⁻²; isolates with [++] medium growth, ability to grow at dilution 10⁻¹, isolates with [+] less growth, ability to grow at 10⁻⁰, [-] isolates cannot grow.

Table 2 shows that all isolates were able to grow well at a concentration of 16% ethanol. Only isolate F08b exhibited good growth at a concentration of 17% ethanol. Meanwhile isolates F01, F08a, F10 and F13 showed average growth and isolates F02 and F13 were still able to grow, but their growth was weak. Most of the yeast isolates were unable to grow at 18% ethanol concentration. Only isolate F01 was able to survive to grow at high ethanol concentrations. Increasing ethanol concentrations can affect yeast tolerance in ethanol [18]. The accumulation of ethanol in the fermentation media can create significant stress on yeast during the fermentation process [19]. High concentrations of ethanol can reduce resistance and it caused yeast cells unable to grow. Ethanol can also affect cellular metabolism and macromolecular biosynthesis by inducing protein production as well as when exposed to high temperatures, lowering RNA levels and protein accumulation, increasing mutation frequency, altering metabolism, denaturing intracellular proteins and glycolytic enzymes [20]. The yeast isolates in this study were tolerant to ethanol because they were able to grow well up to 16% ethanol. The concentration is relatively high according to the real condition of ethanol fermentation. This is in accordance with the result of Tikka et al [21].

3.3. Bioethanol production of thermo-tolerant yeast and ethanol-tolerant yeast from local fermented foods in Indonesia

Ethanol production test was conducted to determine the ability of the isolates obtained in producing ethanol. The results of the ethanol productivity test of each isolate can be seen in Figure 1.
In Figure 1, it can be seen that at 12 hours all isolates were able to produce ethanol but the average productivity was still low. Isolate F08b was the isolate that produced the highest ethanol, which was 2.18% at the 12th hour. While the lowest result was shown by isolate F08a, which was 0.10%. All isolates exhibited an increase in ethanol content from the 12th hour. The highest ethanol yield was shown by isolate F02 of 2.79%, while the lowest was indicated by isolate F08a, which was 0.57%. According to the data, it was found that at 12 to 24 hours there was an increase in ethanol levels. This phenomenon occurred because the yeast in the media has carried out the fermentation process. Isolates F01, F08a, F08c and F10 exhibited an increase in ethanol content at 48 hours with the highest yield indicated by isolate F10 of 3.37%, while the lowest yield was indicated by isolate F08a of 1.21%. Meanwhile isolates F02, F08b and F13 showed a decrease in ethanol content. The longer fermentation time will be able to increase the amount of ethanol content, but after the optimum conditions were reached the bioethanol content decreases gradually, and after the extension of the fermentation process, the ethanol yield will decrease due to the production of other metabolites such as esters or glycerol [22].

The results of the above study on isolates F01, F08a, F08c and F10 showed that the longer the fermentation process was carried out, the greater the concentration of ethanol produced. However, isolates F02, F08b and F13 experienced a decrease in ethanol content at 24 hours. This is in accordance with research conducted by Setiawati which states that the longer the fermentation time, the total sugar of the product decreases and the ethanol content of the resulting product also tends to increase. However, if it exceeds the optimum limit, the ethanol concentration will decrease. This occurred because the longer the fermentation time, the less nutrient or sugar content in the media will result in a decrease in the concentration of bioethanol due to a further reaction from yeast by changing oxidized bioethanol into acetic acid at high temperature [23].

4. Conclusions
All yeast isolates from Indonesian fermented foods were tolerant to temperature of 48°C, while F08a and F08b were tolerant to temperature of 50°C. All yeast isolates were tolerant to ethanol concentrations of 17%, while F01 and F08b were tolerant to ethanol concentrations of 18%. F10 has the highest ethanol production with 3.37% ethanol in 48th-hour fermentation. Molecular identification and further characterization of F08a, F08b, F01, F10 as a potential yeast isolate are required.

References
[1] Banat I M, Nigam P, Singh D, Marchant R, McHale A P 1998 Ethanol production at elevated
temperatures and alcohol concentrations: Part I–yeasts in general. World J Microbiol Biotechnol. 14 809-821

[2] Anwar Z, Gulfraz M, Irshad M 2014 Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy: a brief review. J Radiation Res Appl Sci. 7 163-173

[3] Kosaka T, Lertwattanasakul N, Rodrussamee N, Nurcholis M, Dung N T P, Keo-oudone C, Murata M, Gotz P, Theodoropoulos C, Suprayogi, Maligan J M, Limtong S, Yamada M 2018 Potential of thermotolerant ethanologenic yeasts isolated from ASEAN countries and their application in high-temperature fermentation (London: Intech Open)

[4] Basso L C, de Amorim H V, de Oliveira A J, Lopes M L 2008 Yeast selection for fuel ethanol production in Brazil. FEMS Yeast Res. 8 1155–1163

[5] Nurcholis M, Fernando D, Zubaidah E, Maligan J M 2020 Isolasi dan identifikasi khamir thermotoleran dan ethanol-toleran pada buah lokal Indonesia (Isolation and identification of thermotolerant and ethanol-tolerant yeast on Indonesian local fruits). Jurnal Pangan dan Agroindustri. 8(3) 122-133 [In Indonesian]

[6] Nurcholis M, Kurniawan R, Kusnadi J, Maligan J M 2021 Isolation of thermo-tolerant and ethanol-tolerant yeast from local vegetables and their potential as bioethanol producers. IOP Conf. Series: Earth and Environ Sci. 733 012135

[7] Sumerta I N, Kanti A 2017 Keragaman jenis khamir penghasil etanol yang diisolasi dari makanan fermentasi di Kepulauan Riau (Diversity of ethanol producing yeasts isolated from fermented foods in Riau Islands). Jurnal Biologi Indonesia. 13(1) 61-69 [In Indonesian]

[8] Murata M, Nitiyon S, Lertwattanasakul N, Sootsuwan K, Kosaka T, Thanonkeo P, Limtong S, Yamada M 2015 High-temperature fermentation technology for low-cost bioethanol. J Japan Inst Energy. 94 1154-1162

[9] Chamnipa N, Thanonkeo P, Kusnadi J, Maligan J M 2018 The potential of the newly isolated thermotolerant yeast Pichia kudriavzevii RZ8-1 for high temperature ethanol production. Braz J Microbiol. 49 2 378-391

[10] Keo-oudone C, Nitiyon S, Sothitham P, Tani A, Lertwattanasakul N, Yuangsaard N, Bounphanmy S, Limtong S, Yamada M 2016 Isolation and characterization of thermotolerance ethanol-fermenting yeasts from Laos and application of whole-cell Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF/MS) analysis for their quick identification. African J Biotechnol. 15 6 153-164

[11] Nurcholis M, Nitiyon S, Suprayogi, Rodrussamee N, Lertwattanasakul N, Limtong S, Kosaka T, Yamada M 2019 Functional analysis of Mig1 and Rag5 as expression regulators in thermotolerant yeast Kluyveromyces marxianus. Appl Microbiol Biotechnol. 103 395-410

[12] Tiscione N B, Alford I, Yeatman D T, Shan X 2011 Ethanol analysis by headspace gas chromatography with simultaneous flame-ionization and mass spectrophotometry detection. J Anal Toxico. 35 501-511

[13] Caspeta L, Nielsen J 2015. Thermotolerant yeast strains adapted by laboratory evolution show trade-off at ancestral temperatures and preadaptation to other stresses. mBio 6(4):e00431-15.

[14] Arora R, Behera S, Kumar S 2015 Bioprospecting thermophilic/thermotolerant microbes for production of lignocellulosic ethanol: a future perspective. Renew Sust Energy Rev. 51 699-717

[15] Piper P W, Milson S H, Mollapour M, Panaretou B, Siligardi G, Pearl L H, Prodromou C 2003 Sensitivity to Hsp90-targeting drugs can arise with mutation to the Hsp90 chaperone, cochaperones and plasma membrane ATP binding cassette transporters of yeast. European J Biochem FEBS Press. 270 4689-4695

[16] Choudhary J, Singh S, Nain L. 2016. Thermotolerant Fermenting Yeast For Simultaneous Saccharification Fermentation Of Lignocellulosic Biomass. Electron J Biotechnol. 21 82–92 (2016) 45

[17] Kuroda K, Ueda M 2018 Adaptive evolution of yeast under heat stress and genetic reconstruction to generate thermotolerant yeast. Origin Evolution Biodiversity. 22-36
[18] Dalawai N, Karupa K N, Nadkarni S, Bharani S, Harinikumar K M 2017 Screening of efficient ethanol tolerant yeast strain for production of ethanol. *Int. J. Pure Appl. Biosci.* 5 (1) 744-752

[19] Stanley D, Bandara A, Fraser S, Chambers P J, Stanley G A 2010 The Ethanol Stress Response and Ethanol Tolerance of *Saccharomyces cerevisiae*. *J Appl Microbiol*. 109 (1) 13-24

[20] Hu X H, Wang M H, Tan T, Li J R, Yang H, Leach L, Zhang R M, Luo Z W 2007 Genetic dissection of ethanol tolerance in the budding yeast *Saccharomyces cerevisiae*. *Genetics*. 175 1479–1487

[21] Tikka C, Osuru H P, Atluri N, Raghavulu P C V, Yellapu N K, Ismail S M, Uppu V P, Sudheer A, Narasimha V K, Matcha B 2013 Isolation and characterization of ethanol tolerant yeast strains. *Bioinformation*. 9(8) 421-425

[22] Fitriani M, Sri P 2016 Pengaruh lama fermentasi terhadap kualitas bioetanol pada kulit nangka (*Artocarpus heterophyllus*) (The effect of fermentation time on the quality of bioethanol in jackfruit peel). *Proceeding Seminar Nasional II Biologi, Sains, Lingkungan, dan Pembelajaran, Fenddikan Biologi FKIP Universitas Mulawarman. Samarinda* [In Indonesian]

[23] Febrianti S 2018 Penapisan khamir untuk produksi bioetanol dari nira *Sorghum bicolor* L. (Yeast screening for bioethanol production from *Sorghum bicolor* L.) Thesis. Departemen Biokimia Fakultas Matematika Dan Ilmu Pengetahuan Alam Institut Pertanian Bogor [In Indonesian]