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

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Abstract Bio-ethanol can be obtained from simple sugars, starch-based biomass, or lignocellulosic biomass through fermentation. Conventional yeast acts as a bioethanol producer but has limited tolerance to temperatures above 40°C and high concentrations of ethanol. Therefore, thermo-tolerant and ethanol-tolerant yeast are required because it has many benefits on high-temperature fermentation in the industry. This research is a descriptive study conducted in three stages. The first stage is to isolate yeast from eggplant, cabbage, potatoes, mustard greens, bitter melon, squash, green beans, kale, and celery. The second stage is to test the temperature and ethanol tolerance from yeast that has been obtained. The third stage is to test the ethanol productivity of the isolated yeasts. The results showed isolates S17c, S17d, S17a, S17b, S09, S12, S13a, S13b, S17e, S18a, and S18b grew at 45°C. While in the ethanol tolerance test found that isolates S17c, S17d, S17b, S17a, S09, S12, S13a, S13b, S18a, and S18b grew on YPG agar supplemented with 15% ethanol content. Isolate S17d has the highest ethanol production with 3.13% ethanol in 24th-hour fermentation.

1. Introduction
In order to preserve natural resources and to reduce carbon dioxide emissions from fossil fuels, many researchers focus on the exploration of biofuels as a future alternative fuel. Bio-ethanol is a biochemical liquid produced from biomass through fermentation and has the potential to be applied as an alternative biofuel or bioenergy [1, 2]. Bio-ethanol can be produced from the fermentation process and continued with the distillation. The process is well known to produce the most prominent and cost-effective biofuel [3]. Moreover, many industries attempt to develop new methods, allowing the cost-effective production of bio-ethanol [1]. Recently, industries in tropical countries have some difficulties with bio-ethanol production due to the inability of a conventional yeast strain to grow at temperatures above 40°C and ethanol concentration more than 20% [4]. Since microorganisms are essential for bio-ethanol formation, their attractive characteristics such as tolerance to a high temperature and high concentration of ethanol are required. Most of the ethanologenic yeast, such as Saccharomyces cerevisiae have optimal growth and ethanol production at below 35°C.
In general, most of the microorganisms in the environment tend to be mesophiles. Thermo-tolerant microorganisms were found with optimal growth temperatures of 5-10°C higher than usual mesophilic strains, classified as the same genera or species [5]. Researchers from tropical countries such as Thailand, Vietnam, Indonesia, and Laos obtained thermo-tolerant yeasts [6]. The yeasts are able to grow at high temperatures and can produce ethanol. In the fermentation process at high temperature and high concentration of ethanol, there is a natural selection for obtaining yeast with resistance to some stressors. Yeasts are unable to grow probably due to unfavourable environmental conditions, limited nutrition, or accumulation of metabolic products from the yeast itself. Some of the stress-tolerant yeasts are expected to provide a benefit for the industrial processes due to their resistance to unfavourable conditions and robustness such as inhibition of microbial contamination, reduction of cooling cost, and application of simultaneous saccharification and fermentation [5-7].

In the previous study, thermo-tolerant yeasts were obtained from fruits, flowers, vegetables, fermented foods, plants, soils, and water [6]. Some thermo-tolerant yeast isolates were obtained from local Indonesian fruits such as papaya, banana, star apple, and cacao [8]. However, there is no report of thermo-tolerant yeast from local Indonesian vegetables. In this study, vegetables were selected as the substrate of yeast isolation because it contains simple sugars and micronutrients for yeast growth. This research aimed whether the local vegetables (from eggplants, cabbage, potatoes, mustard greens, bitter melon, chayote, green beans, kale, and celery) have yeast that has temperature tolerant and ethanol tolerant properties. Temperature tolerance property is crucial for the bio-ethanol industry due to some advantages such as reducing cooling costs, preventing contamination [9]. In order to explore thermo-tolerant and ethanol tolerant yeast isolates from Indonesian local vegetables, screening, and further analysis is essential to be investigated.

2. Materials and methods

2.1. Materials
The eggplant, cabbage, potatoes, mustard greens, bitter melon, squash, green beans, kale, and celery were purchased from Tawangmangu market of Malang city. The D-glucose (Sigma), peptone (Kyokuto), yeast extract (Nacalai Tesque), gelatin (Merck), 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), incubator (Binder), laminar airflow (local), spectrophotometer (Unico UV-2100), electric stove (Maspion), digital scale (Metler Denver AA 200), oven (Binder), refrigerator, colony counter brand (WTW BZG 30), vortex, micropipette (Gilson), microtip (blue tip, yellow tip), shaker water bath (Julabo SW22), cold centrifuge (Hettich Zentrifugen Mikro22R), microscope (Olympus Optical Co.Ltd), Gas Chromatography FID (HP-5890), and glassware.

2.3. Yeast isolation
The samples (eggplant, cabbage, potatoes, mustard greens, bitter melon, squash, green beans, kale, and celery) were taken and weighed as much as 5 grams and then dissolved into an Erlenmeyer flask containing 45 ml of sterile distilled water and then homogenized. The YPG broth contains 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) D-glucose. The sample was inoculated into 9 ml of YPG broth and incubated at 30°C for 48 hours. About 1 ml of cultures were taken and inoculated on YPG agar plates (contains 1.5% (w/v) agar) by the spread plate method, and it was incubated at 30°C for 24 hours. The plates were observed, and one loop colony was taken and streaked on YPG agar with the addition of 0.05% (w/v) chloramphenicol using the radian method until obtaining a single colony. The single colony was used for microscopic and macroscopic identification of microorganisms [11] with slight modification.
2.4. Screening of thermo-tolerant yeast
Isolates from Petri dishes were inoculated into 3ml of YPG broth and incubated for 18 hours at 30°C and shaken at 160 rpm. Then the culture was subjected to low-speed centrifugation at 5000 rpm, at 4°C for 5 minutes. The pellets were taken and washed with sterile distilled water and carried out twice. Dilutions were carried out up to $10^{-4}$ and checked OD at 660nm. Each dilution was inoculated using a 5µl micropipette on a cup by using a spotted test [9-10]. The plates then were incubated for 48 hours at 30°C at 37°C, 40°C, 42°C, 45°C, 50°C, 55°C, 60°C to determine the thermo-tolerant yeast.

2.5. Screening of ethanol-tolerance yeast
Yeast isolates were streaked on YPG agar plates with the addition of ethanol at different media concentrations [9]. Ethanol concentration (0%, 1.5%, 3%, 4.5%, 6%, and 7%, 8%, 9%, and 10%) (v/v) continued to be added to the media until the microbes could no longer grow with increasing ethanol added. Isolates that grew on YPG agar containing 10% ethanol were tested again at higher concentration (12%, 15%, 18%, 20%) (v/v), and incubated at room temperature of 30°C for 24 hours to determine the ethanol-tolerance yeast.

2.6. Ethanol production of several yeast isolates
The ethanol production was carried out by taking one single colony of each isolate following the final log phase respectively, then isolated on YP + 10% glucose fermentation medium and incubated at 40°C on a shaker water bath. Ethanol levels were observed at 12, 24, 48 hours. The analysis of ethanol concentration was using Gas Chromatography with simultaneous Flame-Ionization (GC-FID). In short [12] with slight modification, about 400 µl of the sample was added with 200 µl of acetonitrile and homogenized. Then 400 µl sample was injected into the equipment, and acetonitrile value and ethanol levels were measured.

3. Results and Discussion

3.1. Thermo-tolerant yeast from local vegetables in Indonesia
Temperature is one of the factors that influence the growth of microorganisms. Tolerance of temperature is required in the process of ethanol production. It is aimed at the use of broad substrates, higher levels of saccharification and fermentation, minimizing contamination, lower pumping and stirring, reducing cooling costs, fewer energy requirements in product mixing and recovery [13]. Yeast isolation from local vegetables was carried out to obtain thermo-tolerant yeast during bioethanol fermentation. Yeast which is tolerant to temperature was taken from microorganisms derived from several vegetables in local markets. The yeasts were gradually screened at higher temperatures. In this study, we obtained several yeast isolates from five of eleven vegetables, and they could grow at a temperature of 45°C, which can be seen in Table 1.

The thermo-tolerant yeast strains were isolated from soil, and water samples from sugar cane plantations and sugar factories in Thailand grew well at 40°C and 45°C [14]. In addition, thermo-tolerant yeast isolates from local fruits, flowers, and sugarcane in Vietnam could grow up to 45°C, and some yeast isolates from various samples including fermented foods, fruits, and soils in Indonesia were able to grow up to 40°C [6]. According to Nurcholis et al., thermo-tolerant yeast from local Indonesian fruits such as star apple, banana, cacao, and papaya could grow up to 48°C [8]. Some of the thermotolerant yeasts isolated from Thailand were identified as Kluyveromyces marxianus, S. cerevisiae, and Pichia kudriavzevii [15]. Thermotolerant yeasts from tropical countries such as Thailand, Vietnam, Indonesia, and Laos were identified as Candida tropicalis, S. cerevisiae, P. kudriavzevii, Candida glabrata, Torulaspora globosa, Candida nivariensis, Pichia manshurica, Hanseniaspora opuntiae, and Meyerozyma caribbica [6]. To find out the level of tolerance to temperatures, eleven selected isolates from five vegetables were further analysed in the temperature range of 37°C to 60°C, which can be seen in Table 2.
Table 1. Yeast isolates from local vegetables.

| No | Yeast Isolates | Source of Isolate |
|----|----------------|-------------------|
| 1  | S09            | Squash            |
| 2  | S12            | Green Beans       |
| 3  | S13a           | Carrot            |
| 4  | S13b           | Carrot            |
| 5  | S17a           | Kale              |
| 6  | S17b           | Kale              |
| 7  | S17c           | Kale              |
| 8  | S17d           | Kale              |
| 9  | S17e           | Kale              |
| 10 | S18a           | Celery            |
| 11 | S18b           | Celery            |

Table 2. Growth of yeast isolates from local vegetables under various temperatures.

| No | Yeast Isolates | Temperatures (°C) |
|----|----------------|-------------------|
|    |                | 37 | 40 | 45 | 50 | 55 | 60 |
| 1  | S09            | +++| +++| +  | -  | -  | -  |
| 2  | S12            | +++|+++ | +  | -  | -  | -  |
| 3  | S13a           | +++|+++ | +  | -  | -  | -  |
| 4  | S13b           | +++|+++ | +  | -  | -  | -  |
| 5  | S17a           | +++| ++ | +  | -  | -  | -  |
| 6  | S17b           | +++| ++ | +  | -  | -  | -  |
| 7  | S17c           | +++| ++ | +  | -  | -  | -  |
| 8  | S17d           | +++| ++ | +  | -  | -  | -  |
| 9  | S17e           | +++| ++ | +  | -  | -  | -  |
| 10 | S18a           | +++| ++ | +  | -  | -  | -  |
| 11 | S18b           | +++| ++ | +  | -  | -  | -  |

Notes: All the yeast isolates were analysed according to [9-10]. The yeast isolates with (+++) strong growth on YPG agar, indicated by the growth ability at dilution of 10^-3; isolates with (+++) medium growth up to dilution 10^-2; isolates with (+) less growth up to dilution 10^-1; isolates with (-) no growth even spotted test without dilution.

Table 2 shows that all isolates grew at 37°C, 40°C, and 45°C. Isolates of S17c, S17d, S17a, S17b, S09, S12, S13a, S13b, S17e, S18a, S18b, S18b grew up to 45°C. However, at 50°C, 55°C, 60°C, all isolates in the study could not grow on solid media. According to Kuroda and Ueda, heat-shock response (HSP) makes the molecular mechanism in cells more tolerant of temperature [16]. This response makes a specific transcription process that can activate or express the chaperone protein gene code called HSPs (heat-shock proteins) in response to high temperatures. The ability of isolates that are tolerant of temperature depends on the cell membrane. According to Arora et al., temperature tolerant microorganisms can produce different enzymes that function under extreme conditions, so they are still able to metabolize and grow [13].

3.2. Ethanol-tolerance yeast from local vegetables in Indonesia

Screening for ethanol-tolerant isolates was carried out to obtain isolates that resistance to high ethanol concentrations media. According to Hoek et al, high ethanol concentrations can damage mitochondrial DNA. Ethanol-tolerant yeast is able to produce ethanol higher than ethanol-sensitive yeast [17]. Ethanol increased the fluidity in the cell membrane, increased the movement of fatty
acids and protein-membrane binding and membrane permeability. Increased permeability in the plasma membrane causes damage to nucleotides, nucleosides, $\text{Mg}^{2+}$, amino acids [18].

Table 3 shows that the isolates that can grow at 15% ethanol content were S17c, S17d, S17b, S17a, S09, S12, S13a, S13b, S18a, S18b. However, the isolated S17e could not grow at 15% ethanol concentration. The ability of yeast tolerance to ethanol varies depending on the strain and composition of the plasma membrane in the form of unsaturated fatty acid, and fatty acyl plays a role in maintaining the integrity of the yeast cell membrane. *S. cerevisiae* can grow up to 16.5% ethanol concentration. *S. cerevisiae* exhibited strong resistance to ethanol compared to that of other yeast strains [19]. The mechanism of ethanol-resistance is related to a complex network at the genome level, as well as multiple interactions of signal transductions pathways and regulatory networks. Transcription dynamics and profiling findings of key gene sets, including heat shock proteins, provided understanding into the tolerance mechanism [20].

### Table 3. Growth of yeast isolates from local vegetables under various ethanol concentrations.

| No | Sample Code | Ethanol concentrations (%) | 4.5 | 6   | 7   | 8   | 9   | 10  | 12  | 15  | 18  | 20  |
|----|-------------|-----------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1  | S09         | +++                        | +++ | +++ | +++ | +++ | +++ | +   | +   | -   | -   | -   |
| 2  | S12         | +++                        | +++ | +++ | +++ | +++ | +++ | +   | +   | -   | -   | -   |
| 3  | S13a        | +++                        | +++ | +++ | +++ | +++ | +++ | +   | +   | -   | -   | -   |
| 4  | S13b        | +++                        | +++ | +++ | +++ | +++ | +++ | +   | +   | -   | -   | -   |
| 5  | S17a        | +++                        | +++ | +++ | +++ | +++ | +++ | +   | +   | -   | -   | -   |
| 6  | S17b        | +++                        | +++ | +++ | +++ | +++ | +++ | +   | +   | -   | -   | -   |
| 7  | S17c        | +++                        | +++ | +++ | +++ | +++ | +++ | +   | +   | -   | -   | -   |
| 8  | S17d        | +++                        | +++ | +++ | +++ | +++ | +++ | +   | +   | -   | -   | -   |
| 9  | S17e        | +++                        | +++ | +++ | +++ | +++ | +++ | +   | +   | -   | -   | -   |
| 10 | S18a        | +++                        | +++ | +++ | +++ | +++ | +++ | +   | +   | -   | -   | -   |
| 11 | S18b        | +++                        | +++ | +++ | +++ | +++ | +++ | +   | +   | -   | -   | -   |

**Notes:**
All the yeast isolates were analysed according to [9-10]. The yeast isolates with (+++) strong growth on YPG agar, indicated by the growth ability at dilution of $10^{-2}$; isolates with (+) medium growth up to dilution $10^{-4}$; isolates with (-) less growth up to dilution $10^{-6}$; isolates with (--) no growth even spotted test without dilution.

### 3.3. Bioethanol production of thermo-tolerant and ethanol-tolerant yeasts

Ethanol fermentation is a biological process where simple sugars such as glucose, fructose, and sucrose are converted into cellular energy and the production of metabolic waste in the form of ethanol and carbon dioxide. At 12 hours, isolates S17b, S17c, S13, s12, s09, s18b, s17d, S13b, S17e, s17a, s18a haven’t produced ethanol. According to Su et al., aeration influences the level of ethanol produced, since, in aerobic conditions, ethanol is not optimally formed because cells prefer to use more substrates for growth than produce ethanol [21]. At 24 hours, isolates with code S17b, S17c, s13, s09, S17d, S13b, S17e, S17a produced ethanol. According to Bestari et al., at different times, there is an increase in ethanol levels resulting from the fermentation process because it increases a lot as the microbial activity experiences growth by multiplying so that the alcohol produced increases [22]. Bio-ethanol can be produced from the fermentation of several raw materials or biomass such as sugar sources, starch sources, or lignocellulosic biomass [23]. At 24 to 48 hours, ethanol levels in isolates S17b, S17c, S13, S17d, S13b, S17a were found to have a decrease in ethanol levels. The ethanol concentration will be even greater during the longer fermentation time, but after the optimum limit, the ethanol concentration will decrease gradually. It happened probably due to the conversion of ethanol to become other substances such as aromatic esters. According to Morrisey et al., *K. marxianus*, thermo-tolerant yeast could produce natural aromatic compounds such as acetate esters during fermentation [24]. The ethanol yield of the S17d (thermotolerant yeast...
from Indonesia) was lower compared to that of other well-known yeast isolates in a previously published paper. *K. marxianus* DMKU-3 (thermotolerant yeast from Thailand) and *K. marxianus* BUNL-21 (thermotolerant yeast from Laos) had ethanol yield of 0.36 and 0.42 g ethanol per g glucose, respectively, at a temperature of 40°C after 24 h fermentation [11]. However, S17d isolate had 0.31 g ethanol per g glucose at the same temperature and incubation time.

![Figure 1. Bioethanol production of thermo-tolerant and ethanol-tolerant yeasts from local vegetables.](image)

4. Conclusions

All yeast isolates from local vegetables were tolerant to the temperature of 45°C while only yeast isolates were unable to grow at an ethanol concentration above 15%. Isolate S17d derived from kale has potential due to its ability to grow both in high temperature and high concentration of ethanol. The isolate could produce 3.13% ethanol at 24 hours fermentation. Further studies such as molecular identification of S17d as a potential yeast isolate is required.

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