Molecular Identification and Potential Ethanol Production of Long-term Thermo-tolerant Yeast *Candida Tropicalis*

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**Abstract.** *Candida tropicalis* is an organism capable of promoting the fermentation process of starch media supplementation with α-amylase sufficient to produce ethanol. This study aims to identify *Candida tropicalis* yeast and the potential for ethanol production which is tolerant at high temperatures. Molecular identification methods using Molecular rDNA sequencing and measurement of ethanol production at the incubation rate of yeast growth at 38°C, 39°C and 40°C. The best isolates used were JCBI-23 and JCBI-24 from Indonesia and DMKU-3 thermo tolerant yeast control isolates *Kluyveromyces marxianus*. The results showed that molecular identification of JCBI-23 isolate was *Candida tropicalis* culture CBS: 2424 large subunit of ribosome RNA gene with an identification value of 99% and JCBI-24 isolate was *Candida tropicalis* isolate 4kr27 26S ribosome RNA gene with an identification value of 96%. Based on the growth resistance test, isolates showed that optimum growth occurred during seven-day incubation period and tend to decrease its growth on day 14, both at 37°C, 38°C and 39°C. The best ethanol production at 37°C and not significantly different from the controlled isolates, and showed ethanol production tend to be constant on day 48, both at 37 and 40°C.

**Keywords:** Thermo-tolerant Yeast, *Candida tropicalis*, bimolecular identification, and ethanol production

1. **Introduction**

The main substitution of oil-based fuels is needed for energy security and climate change considerations. Although future bio-fuel ethanol will certainly require cellulose technology, starch is still a major substrate that is widely used [1]. Cellulose technology requires bioconversion of lignocelluloses’ biomass into ethanol which is environmentally friendly from the management of waste material and as an alternative to sustainable green energy production. There are three main stages in the second generation of bio ethanol, namely 1) initial test of lignin residues, 2) hydrolysis of cellulose and hemicelluloses, and 3) fermentation of sugar. The initial test process aims to separate lignin and decompose the lignocelluloses structure so that this process is quite expensive for the conversion of biomass and lignin which has difficult fermentation properties [2]. For this reason, it is necessary to simplify the saccharification process to make costs more efficient and choose potential isolates due to their ability to degrade starch in fermentation processes that are tolerant at high temperatures. It was found that many microorganisms, including *Saccharomyces cerevisiae*, were unable to degrade starch because they did not produce starch decomposing enzymes as α-amylase, β-
amylase, pullulanase or isoamylase, and glucoamylase [3]. It is necessary to either add starch decomposing enzymes to the starch before fermentation or to use recombinant strains that produce starch decomposing enzymes to utilize this carbon source [3][4][5]. Starch fermentation with S. cerevisiae transformed with the α-amylase and glucoamylase genes showed some ethanol productivity similar to that observed when decomposition enzymes of starch were added to the media [6][7][8][9]. The results of the research show that Yeast Candida tropicalis has a considerable metabolic capacity on various types of carbon and as the only source of carbon and energy, biomass producer is quite high and pure oxidative metabolic ability in conditions of growing medium supplied sufficient oxygen [10]. The results showed that Candida tropicalis isolates had the ability of hydrolysis and cellulose fermentation to produce ethanol through the metabolic ability of monosaccharide glucose, but not for pentose monosaccharides such as arabinose and xylose [11]. Candida tropicalis is known to produce ethanol from starch, although at a low level, because of glucoamylase production [5]. Previous studies have studied the feasibility of producing ethanol from glucose by this yeast both when cells are free or immobilized in calcium alginate [12]. The results of subsequent studies show that Candida tropicalis has the ability to tolerate and decompose both phenols and polyphenols at a high toxic concentrations to bacteria and other yeast species [13][14] and become an attractive system to produce biofuel from renewable sources. The unique enzyme activity, osmotolerant, has a wide temperature range are the basic properties of this yeast in food processing. Besides having the potential for ethanol production from lignocelluloses’ biomass, its role can also replace Saccharomyces cerevisiae yeast in the fermentation process. The genus Candida contains asexual Ascomycetes species that have not been clarified and some species are anamorphs from teleomorphs (sexual or perfect species) originating from different genera.

Candida is widely distributed in natural and artificial habitats and has been used in food management for a long time, including wine production and fermentation of food and traditional drinks. The fermentation results of Candida tropicalis isolates at 140.1 g/L using glucose 50 g/L were not significantly different from using Saccharomyces cerevisiae isolates of 148.6 g/L [15]. In line with the results of previous studies showed that starch melting alone is sufficient to encourage the fermentation of starch to ethanol by Candida tropicalis YMEC14 which achieves ethanol comparable to that obtained by using the strain Saccharomyces cerevisiae which displays both α-amylase and glucoamylase [16].

The optimum growth of Candida tropicalis isolates at 37 °C and pH 7, and produced maximum enzymes for 5 days of incubation period, while cellulose activity was found at 40 °C and pH 8 so that these isolates could be utilized for cellulose biotechnology because it produces extracellular enzymes [17]. To increase the utilization of this carbon source and increase the rate of alcohol production, starch need to dissolve with α-amylase. This study aims to identify and measure the optimum potential of Candida tropicalis isolates based on the tolerance level at high temperatures and the optimal amount of ethanol produced. As a comparison, isolates using yeast Kluyveromyces marxianus which produces ethanol comparable to Saccharomyces cerevisiae and has a growth resistance up to 45 °C [18].

2. Research Methodology

2.1. Material and method
The growth medium used is YPD (1% Yeast Extract, 2% Peptone and 2% Glucose, and 1.5% agar). The research isolates used were JCBI-23 and JCBI-24. Growth resistance test for isolates with incubation per two weeks of observation starting from a temperature of 38 °C 39 °C and 40 °C agitation 160 rpm with 10 observation samples and 5 ml media volume. Identifying the isolates so that the phylogenetic tree is known based on the results of the sequencing D1/D2 domains in rDNA. Ethanol test uses YPD media (glucose 16%) at 37 °C and 40 °C with measurement periods of 6, 12, 24, 36 and 48 hours. Control isolates for ethanol test using DMKU3-1042 thermo-tolerant yeast of Kluyveromyces marxianus.
2.2. Genomic DNA extraction and sequencing of 26S rDNA
Media growth and cultural conditions are the same as those described previously. In short, genomic DNA extraction, purification, polymerase chain reaction (PCR) and DNA sequencing reactions are carried out as follows: cells washed once with distilled water and re-suspended in 2 mL of distilled water. One millilitre of cell suspension collected in a 1.5 mL micro centrifuge tube. After centrifugation, remove the excess water, and cells stored in freezer (-20 ºC) until it used. Genomic DNA is separated and purified using DNA extraction tools (Takara, Japan). Domain sequence of D1/D2 rations of 26S rDNA carried out on PCR products of genomic DNA fragments, extracted from yeast cells. The D1/D2 domain of rDNA amplified by PCR with the primary forward NL-1 and NL-4
forward primary NL-1: 5’-GCATATCAATAAGCGGAGGAAAAG-3’. Reverse primary NL-4: 5’-GTCCGTGT TTCAAGACGG-3’ (O’Donnell K. in Reynolds DR, Taylor JW, 1993) [21]. PCR products were examined by Agarose gel electrophoresis, purified using ABI Big Dye terminator Cyclo sequencing Kit Version 3.1 (Applied Bio systems, California, USA) with external primers IL-1 and IL-4 [19]. The sequence determined by ABI PRISON BIO Analyzer (Applied Bio systems) according to the manufacturer's instructions. Pair-wise comparing order uses homologous localized localization search (BLAST) tool.

2.3. Analysis of fermentation parameters
Cell growth determined by measuring the optical density at 660 nm in a spectrophotometer (Spectrophotometer 258, Corning, New York, USA), after washing it twice with distilled water. The concentration of ethanol analyzed by gas chromatography (Shimadzu GC-9A, Shimadzu, Kyoto, Japan) using a polyethylene glycol (PEG20 M) packing column (length 2.1 m, OD 5 mm, ID 3.2 mm), nitrogen as a carrier gas (35 ml/min), and the ionization detector is on (injection temperature of 200°C, oven temperature at 180°C, detector temperature at 200°C). Sugar as a carbon source in the media was analyzed using high-performance liquid chromatography (Hitachi, Japan) with GLC610-S Gel (Hitachi) pack column connected to the refractive index detector Model L-2490 (Hitachi) in 0.5 ml/millilitre water mode deionization at 60°C [22].

3. Research Results and Discussion

3.1. Identification of Isolates
Identification of isolates included genomic isolation, PCR sequencing, and DNA purification. The results of electrophoresis genomic isolation went well and there was a band.

![Figure 1](image_url) (a) The electrophoresis from the isolation of the genome were quite good with the appearance of the band although quite thin both in the JCBI 23 and JCBI 24 isolates. (b) PCR sequencing showed a fairly sharp band of the two test isolates, and (c) DNA Purification Results also shows a clear band so DNA identification can be continued.

Figure 1 (b) shows that all isolates can be done on PCR which are marked by the presence of the band, so that the next experiment, DNA purification, can be done and the results are shown in Figure 1 (c). Identification of isolates run quite well so that they can be continued with DNA sequencing.
3.2. Genomic DNA Extraction and Sequencing of 26S rDNA
The results of genomic DNA extraction and sequencing of the two strains used were identified in the following table:

| No. | Strains | Identification D1/D2 | TOF/MS |
|-----|---------|----------------------|--------|
| 1   | JCBI-23 | *Candida tropicalis* culture CBS: 2424 | C.tropicalis |
| 2   | JCBI-24 | *Candida tropicalis* isolate 4kr27 26S | C.tropicalis |

In general, the identification shows the same yeast species - tropical *Candida*, so this identification is very valuable as a native Indonesian yeast species to be tested for its superiority as an important organism in the fermentation process.

3.3. Yeast Thermo-tolerant Test from JCBI Isolates in Various Temperature Conditions
Test of the potential of yeast *Candida tropicalis* against temperature in YPD media at four temperature levels is shown in Table 2 below:

| Strains | Sample | Temperature (°C) |                |
|---------|--------|----------------|---------------|
|         |        | 37 | 38 | 39 | 40 |
| JCBI-23 | 1      | +++ | +++ | ++ | +  |
|         | 2      | ++  | +  | +  | -  |
|         | 3      | +++ | ++ | ++ | ++ |
|         | 4      | +++ | +++| ++ | -  |
|         | 5      | +++ | +++| ++ | +  |
|         | 6      | +++ | +++| ++ | -  |
|         | 7      | +++ | +++| ++ | ++ |
|         | 8      | +++ | ++ | +  | -  |
|         | 9      | +++ | +++| ++ | -  |
|         | 10     | +++ | +++| ++ | ++ |
| JCBI-24 | 1      | +++ | ++ | +  | -  |
|         | 2      | +++ | +++| ++ | -  |
|         | 3      | +++ | +++| ++ | +  |
|         | 4      | +++ | +++| ++ | +  |
|         | 5      | +++ | ++ | +  | -  |
|         | 6      | +++ | +++| ++ | -  |
|         | 7      | +++ | +++| ++ | -  |
|         | 8      | +++ | ++ | +  | -  |
|         | 9      | +++ | +++| ++ | -  |
|         | 10     | +++ | +++| ++ | -  |

Description: +++ = Strong growth, ++ = Medium Growth, + = Less Growth, - = Not Growth

The optimal growth resistance of yeast *Candida* at 38°C, however at the next temperature, yeast growth decreased to 40°C. The results of the study in Table 2 show that at 37°C *Candida* yeast has a very good growth of 100%, at a temperature of 37 and 38°C, and is capable to grow at 35% at 40°C. This shows that *Candida* yeast is able to survive up to a maximum temperature of 40°C. In accordance with the opinion of Sadaf S. and A. Rehman, that optimum plant yeast *Candida* at a temperature of 37°C and able to survive to a temperature of 40°C, where at these temperatures the yeast is able to...
live along with the optimal temperature for cellulose activation [17]. Growth Resistance Test for *Candida tropicalis* isolates can be seen in the following figure:

![Figure 2](image)

**Figure 2.** (a) Effect isolates JCBI-23 was growth in YPD medium 2% at 37, 38, 39 and 40°C. At the time indicated, the OD value have been measured for 7 and 14 days, and (b) for isolate JCBI-24.

In graph 2, it can be seen that the growth of yeast from the JCBI 23 and JCBI 24 strains for figure 3 shows that from the 4 temperature levels, the maximum growth occurs at the age of 7 days of growth, but in the following day there is a decrease as seen in the growth on day 14th. The temperature level used shows that the highest growth of *Candida tropicalis* yeast at a temperature of 38°C, then the increase in temperature further decreases yeast growth potential even at 40°C, this type of yeast can still grow.

### 3.4. Result of ethanol fermentation

Ethanol test results showed that JCBI-23 and JCBI 24 isolates at 37°C reached optimum ethanol concentration to 24 hours, while in other time, it tends to provide more stable growth as shown in Figure 4. The concentration of ethanol produced by these isolates is not different from DMKU isolate as the control. This shows that the posture of Candida tropicalis yeast to produce ethanol concentration is not significantly different from Kluyveromyces marxianus yeast, where yeast has more ability to survive its growth to 45°C which produces ethanol comparable to Saccharomyces cerevisae [18]. Indirectly, the ability of Candida tropicalis yeast to produce ethanol is comparable to Saccharomyces cerevisiae.

![Figure 3](image)

**Figure 3.** (a) Ethanol production from JCBI 23 and JCBI 24 isolates as Candida tropicalis yeast with DMKU-3-comparing yeast identified as Kluyveromyces marxianus yeast at 37°C. (b) at 40°C.
Figure 3 (b) also shows the same tendency to obtain the results of ethanol concentration of JCBI 23 isolates and JCBI 24 with DMKU-3 comparator isolates at 40°C. The ability of these isolates to produce ethanol were because Candida tropicalis capable to carry out two main processes in converting cellulose to ethanol, namely hydrolysis of cellulose degradation into glucose, and fermentation of the conversion process of glucose to bio ethanol [11]. Candida tropicalis has the advantage of bio ethanol fermentation such as having a higher growth rate than S. cerevisiae, and able to grow in arabinose or xylose as a single carbon source [11]. Cellulose is one of the most important component in lignocelluloses biomass and can be converted to ethanol. Fermentation for 1 day (24 hours) showed that Candida tropicalis were able to hydrolyze and ferment cellulose to bio ethanol as shown in Figure 4a and Figure 4b, and the average concentration of ethanol as below.

Figure 4 (a). Average ethanol concentration from NCBI 23 and JCBI 24 isolates and isolates DMKU comparison at 37°C, and (b) at 40°C

Figure 4a. shows that the concentration of ethanol from JCBI 23 and JCBI 24 isolates tends to be greater than DMKU-3 isolates. Whereas at 40°C, the DMKU-3 isolates compared was higher than JCBI 23 and JCBI 24. In general, the average concentration of ethanol produced both by JCBI 23 and JCBI 24 isolates and DMKU comparator isolates was not much different, as shown in Figure 4b. The occurrence of an average decrease in the results of ethanol concentration due to the temperature rises from 37°C to 40°C shown in Figure 4b, because of the presence of toxic substances in hydrolyzed which might interfere sugar catabolism [23]. Acetic acid is an important fermentation inhibitor that reduces the rate and yield. The toxic effect is basically because it is not dissociated. It was reported in the literature [24], that the inhibition of acetic acid for ethanol production was highest at pH 5. In previous studies, it was shown that C. tropicalis has the advantage of fermentation of bio ethanol production such as having a higher growth rate than S. cerevisiae, and capable of grown in arabinose or xylose as a single carbon source [11]. C.tropicalis is known to produce ethanol from starch in low levels [12], but further experiments state that C.tropicalis is a promising agent for producing ethanol from renewable sources [13][14]. The advantage of using C.tropicalis in the fermentation of starch to ethanol is the a necessary process to perform saccharification steps and the process of producing ethanol around 56 g/L. This produces ethanol similar to fermentation using recombinant S. cerevisiae which expresses amylase and glucoamylase [12], and C.tropicalis yeast has the ability to convert cellulose into ethanol without the saccharification stage. C.tropicalis isolates are able to hydrolyze and ferment cellulose to produce bio ethanol. Bio ethanol is produced by bio-conversion through metabolism of glucose monosaccharides, but not for pentose monosaccharides such as arabinose and xylose. As a major element of lignocelluloses material, these results indicate that C.tropicalis can be used as a microbial agent to bio-converting lignocelluloses biomass into ethanol. [11].

4. Conclusion
Identification of the growth resistance test and ethanol fermentation process on the use of JCBI 23 and JCBI 24 isolates and DMKU-3 comparator isolates concluded that molecular identification of JCBI-23
isolates was Candida tropicalis culture CBS: 2424 large subunit RNA gene ribosome with values of 99% identification and JCB1-24 isolates were Candida tropicalis isolates 4kr27 26S ribosome RNA gene with 96% identification value. The growing resistance of isolates showed that optimum growth occurred during the seven-day incubation period and tend to decrease its growth on day 14th, both at 37°C, 38°C, and 39°C. The best ethanol production at 37°C and not significantly different from the control isolates, and showed that ethanol production tend to be constant on day 48th, both at 37°C and 40°C. This shows that Candida tropicalis isolates have significant potential with superior yeast Kluyveromyces marxianus so that it becomes potential yeast because they can grow to a temperature of 40°C and provide ethanol potential which is not much different from the yeast Kluyveromyces marxianus.

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