Molecular identification of inulinolytic yeast isolated from cherry fruit (*Muntingia calabura L.*) based on internal transcribed spacer sequence

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Abstract. Inulinolytic yeast is a yeast that produces inulinase enzyme (E.C.3.2.1.7). Inulinase has an important role to hydrolyze inulin into simpler molecules that are widely used in industry. Previous research has obtained K4 inulinolytic yeast from cherry fruit (*Muntingia calabura L.*) which has not been identified yet. This study aims to molecularly identify and phylogenetic analysis of K4 inulinolytic yeast isolated from cherry fruit (*M. calabura L.*) based on the Internal Transcribed Spacer (ITS) sequence. Research stages consist of DNA isolation, amplification of the ITS rDNA sequence, electrophoresis of PCR product, analysis of sequence data, and phylogenetic analysis using MEGA X. The results showed that yeast DNA was successfully obtained with a concentration of 807.1 ng/µL and had a purity value of 2.12. Amplification of the ITS rDNA sequence produced amplicon with length ±250 bp. Molecular identification based on homology analysis with database of The National Center for Biotechnology Information (NCBI) and phylogenetic analysis indicated that K4 isolate was the most closely related to *Diutina rugosa* isolate S217 (MF797783).

1. Introduction

Indonesia is a tropical country which has an abundance of biodiversity of flora, fauna, and microorganisms [1]. Recently, the biodiversities in Indonesia that have not been exploited yet, especially microorganisms that have an essential role for human, included yeast. Inulinolytic yeast is a unicellular fungus which can produce inulinase enzyme. Inulinase enzyme is one kind of enzyme that can hydrolyze inulin to be small units such as fructose and fructooligosaccharide (FOS). Inulase is needed in the sugar industries.

Researcher has been isolated inulinolytic yeast from Cherry fruit (*Muntingia calabura L.*) namely K4 isolate, this potential isolate can produce 0.95275 IU/mL of inulinase enzymes [2]. Previous studies, K4 isolate was identified based on morphology and biochemistry. Nevertheless, it may contribute to frequent errors in the accurate identification of species or strain. It must be conducted with identification based on molecular approach.

Molecular identification is an accurate method for analyzing isolate up to the strain level based on DNA sequences analysis (Deoxyribose Nucleic Acid). Molecular identification in yeast can be done by analyzing the Internal Transcribed Spacer (ITS) sequence. Internal transcribed spacers are part of ribosomal RNA (rRNA) which have the highest probability of distinguishing closely related species [3]. The ITS region can be used for yeast identification, the ITS gene is located between 18S and 28S rRNA.
which consists of internal non-coding regions ITS1, ITS2, and the 5.8S rRNA gene [4]. There are some of the advantages of using ITS molecular marker in identifying species of fungi, include; (1) ITS sequences have a high conserved region, (2) ITS region has sequences with high genetic variability, and (3) the presence of ITS sequences in the yeast very abundant, so it can increase the sensitivity of the PCR reaction.

The research efforts to analyze and determine the type of K4 isolate as well as their genetic relationship based on the Internal Transcribed Spacer (ITS) sequence using molecular identification technique.

2. Materials and methods

2.1. Yeast inoculation
The yeast sample (K4 isolate) was grown on inunase selecting medium (ISM) that contain of; 0.5 g yeast extract, 2 g glucose, 1 g peptone and 1.6 g agar into 100 mL of aqua dest. The inoculation was carried out by streak on the medium. Then, yeast culture were incubated for 48 hours in the 37 ºC.

2.2. DNA isolation of K4 yeast
DNA isolation of K4 isolate using chelating ion exchange (Chelex 100) method [5]. The colony of yeast was taken using an inoculating loop then it was put into microtube that already contain of 100 µL ddH2O. Sample was added 100 ml phosphate buffer saline (PBS) 1x. The sample was incubated in 4ºC until 24 hours (overnight). After that, sample was centrifuged at 12,000 rpm for 10 minutes. Supernatant formed was discarded, whereas pellet was added PBS 1 mL. The pellet was centrifuged at 12,000 rpm for 5 minutes. Supernatant was removed carefully and the pellet was added 100 µL of ddH2O and 20% of 50 mL of chelex solution. The sample was incubated on water bath for 10 minutes and homogenized every 5 minutes. The sample was centrifuged at 12,000 rpm for 10 minutes. The supernatant was taken and separated from the pellet. The supernatant which contains of genome DNA was moved into a new microtube. DNA sample was used for amplification stage.

2.3. Amplification of ITS region
Yeast DNA amplification was carried out by making a 25 µl of PCR mix consist of; 12.5 µl My Taq™ HS Red Mix PCR kit, 7.5 µl ddH2O, 1.5 µl ITS4 (10 pmol), 1.5 µl ITS5 (10 pmol) and 2 µl of yeast DNA genome extract as template. Amplification was done using reverse primer ITS4 (5’ – TCC TCC GCT TAT TGA TAT GC – 3’) and forward primer ITS5 (5’ – GGA AGT AAA AGT CGT AAC AAG G –3’). The DNA amplification was carried out at 35 cycles. The optimization process of PCR is performed using PCR gradient technique. PCR stages consist of a pre-denaturation at 95 ºC, 3 minutes; denaturation at 95 ºC, 30 seconds; Annealing phase at 55 ºC, 1 minute; Extension phase at 72 ºC 1 minute; Post extention phase at 72 ºC, 10 minutes. PCR produces are stored at -20 ºC. Electrophoresis was conducted by agarose gel electrophoresis 1%. Agarose gel dissolving with TAE 1x solution. The electrophoresis was carried out using electroporator with a voltage of 100 volts and a time 35 minutes. Then, the DNA band which have formed on the agarose gel are analyzed using GelDoc Uvitec.

2.4. Sequence analysis
Sequencing was conducted by amplifying ITS4 and ITS5 primers of 50 µl total volume in the PCR microtube. Samples are sent to the PT. Genetika Science Indonesia for the sequencing process. After that, the sequencing results are analyzed using BioEdit application and converse to the FASTA format. Then, the sequences were used to search the homology sequences in the National Center for Biotechnology Information (NCBI) databases using BLAST (Basic Local Alignment Search Tool).
2.5. Phylogenetic analysis
The Phylogenetic analysis was performed using Molecular Evolutionary Genetics Analysis (MEGA X) [6]. The DNA sequences that were obtained based on the closest homology are analyzed and compared with the sequence type strain in the GenBank. Phylogenetic tree was reconstructed using neighbour-joining method, whereas genetic distances were calculated using Kimura’s 2 parameters [7].

3. Results and discussions

3.1. DNA isolation of K4 yeast
Molecular identification of K4 yeast based on ITS sequence was performed. Table 1 showed the result of K4 yeast DNA isolation.

| Sample Name | DNA concentration (ng/µL) | λ_{260} | λ_{280} | Purity of DNA (λ_{260}/λ_{280}) |
|-------------|---------------------------|---------|---------|-------------------------------|
| K4          | 807,1                     | 16,142  | 7,598   | 2,12                          |

DNA isolation results were showed that DNA of K4 yeast has a high concentration and high purity. The yield of DNA was claimed as a pure DNA when the ratio of purity value between 1,8-2,0 [8]. DNA purity values of more than 2.0 indicate that DNA was contaminated by RNA [9]. The presence of RNA contaminants is due to the DNA precipitation process that has not been carried out optimally. It is important to carry out this stage very carefully because it can cause an increase of RNA contaminant to DNA. Afterward, the chelex solution also has a vital role in this stage because when the work of the chelex solution was not optimal, it can also lend a negative effect to the results of DNA purity. Before using that reagent in the extraction process, it will be better if the chelex solution was incubated or stored at 4°C to keep the quality of the chelex solution. In the DNA extraction process, cellular component along with DNA and RNA will dissolve in the chelex solution, whereas DNA and RNA will be on the surface of the column. After the centrifugation process, DNA and RNA will remain on the surface while other cellular components are bellow [5]. The purification of DNA sample from RNA contaminants can be done by adding RNAse [10], but in the stage of yeast DNA isolation using the chelex method, RNAse was not added. Based on the analysis of the purity and concentration of K4 yeast DNA, the isolation of DNA using the chelex method was successful and could be continued at the DNA amplification stage.

3.2. Amplification of ITS sequence
Molecular identification has been carried out by analyzing the of ITS region on the K4 yeast DNA. In this stage, the primer pairs are ITS4 as a reverse primer and ITS5 as a forward primer. DNA amplification produced amplicon with a length of ± 250 bp. These results indicate that the ITS region contained in the K4 yeast rDNA was partially amplified by the primary ITS4 and ITS5. The size of yeast ITS region are 450-700 bp [11].

The occurrence of partial amplification is probably caused by several factors that have been supported and confirmed by other researchers. First, the results of yeast DNA isolation using the chelex method have inconsistent qualities that cause the Amplification stage to be suboptimal [12-15]. The inconsistent results may occur due to the chelating group of chelex reagents which are dissolved in the results of yeast DNA extraction, thus binding to the Mg²⁺ ions (cofactor of the DNA polymerase enzyme). The binding of these Mg²⁺ ions can decrease the performance of the DNA polymerase enzyme and will cause a decrease in the PCR sensitivity [16]. Second, primary concentrations are too high. Thus causing primary dimers [14]. According to Innis and Gelfand (1990), high primary concentrations can
cause mispriming, as well as causing reaction between primers which can produce dimeric products. [17] Annealing temperature also affects these pcr results [18]. Inappropriate annealing temperature can cause the ITS primer not to amplify the yeast DNA strand during the PCR process. There is an important factor in the annealing stage, \( T_m \) or melting temperature. \( T_m \) is the most important part of PCR. \( T_m \) is the temperature needed for the primer to attach to the DNA target [19]. The annealing temperature that has been used to amplify K4 yeast DNA was 55 °C, the annealing temperature probably not be appropriate for K4 yeast DNA. \( T_m \) values that are too low can cause the detection of products that are not specifically due to base incompatibility, while \( T_m \) that are too high at the annealing stage can cause PCR product results to be incompatible with the targeted sequence [20].

![Figure 1](image.png)

**Figure 1.** The results of ITS amplification of K4 yeast isolate with Gel Doc compared to 1 kb and 100 bp markers (Geneaid), 2= band of K4 DNA

3.3. Sequencing result and phylogenetic analysis

The ITS sequences that have been amplified are used in homology analysis using BLAST. Table 2 Showed the results of homology analysis based on ITS region.

| Isolate Code | Accession Number of GenBank | Similarity | Closely related species |
|--------------|-----------------------------|------------|------------------------|
| K4           | MF797783                    | 96.30%     | Diutina rugosa isolate S217 |

K4 yeast isolate is different species from the comparative isolate in the NCBI database, because it has a 96.30% of the similarity score or less than 99%. The minimum accepted of homology value for identic species was 99%. The low similarity value of the comparative isolate in the GenBank which shows that the K4 isolate obtained is a new taxon that is closely related to *D. rugosa*.

Based on the results of homology analysis, K4 yeast is a type of Ascomycetes yeast, belonging to the genus of *Diutina* and has similarities with the closest taxon *D. rugosa*. The synonym of *D. rugosa* is *Candida rugosa* [21]. *D. rugosa* is a yeast that has pseudohyphae, this yeast is able to grow aerobically in a medium that has a high carbon and nitrogen source. *D. rugosa* has the ability to produce lipase [22]. *D. rugosa* also able to produce lipase enzymes that have been widely applied in industry [23]. *D. rugosa* (Syn. *C. rugosa*) is a yeast that has been found in feces, streams, butter and also found in the insects [24]. *D. rugosa* is also found in the leaf tissue of rice plants [25]. This yeast is not known as yeast that
can produce inulinase, but k4 yeast isolate which in this study proved to be closely related to *D. rugosa* (Syn. *C. rugosa*), K4 isolate was able to produce inulinase with an average production of inulinase of 0.95275 UI / mL [2]. Table 3. Shows the morphological and biochemical comparisons of K4 isolate with *D. rugosa* (Syn. *C. rugosa*).

**Table 3.** Morphological comparison of K4 isolate with *D. rugosa* (Syn. *C. rugosa*)

| Characteristics       | K4 isolate | *D. rugosa* [24, 26] |
|-----------------------|------------|----------------------|
| Color                 | White      | White-Cream          |
| Surface               | Smooth     | Smooth               |
| Texture               | Butyrous   | Butyrous             |
| Shape                 | Circular   | Circular – Ovoid     |
| Margin                | Entire     | Entire               |
| Asexual Reproduction  | Budding    | Budding              |

Phylogenetic trees were constructed using MEGA X software [6]. Some of the closest ingroups from *D. rugosa* were chosen, including; (1) *C. parapsilosis* (LC390057), (2) *C. metapsilosis* (LC389302), (3) *C. tropicalis* (MF797764), (4) *C. albicans* (MF797779). Outgroups are selected sequences that are closely related to *D. rugosa*, *Saccharomyces cerevisiae* (NR111007). Genetic distance was calculated using Kimura’s 2-Parameter method integrated in MEGAX. Figure 2. shows the results of the phylogenetic reconstruction of K4 isolate.

![Figure 2. Phylogenetic analysis of K4 isolate](image)

The genetic relationship between k4 isolate and some species of *candida* genus were determined from the phylogenetic tree with the bootstrap value that showed the similarity among species. K4 isolate was more closely related to *D. rugosa* (MF797783). The bootstrap value between K4 isolate and *D. rugosa* were around 100%. It means that the branch between K4 and *D. rugosa* isolate was formed 1000 times in phylogenetic tree reconstruction. High bootstrap values can increase the level of trust in phylogenetic tree topologies [27].

Based on the phylogenetic analysis above, it can be seen that the scale substitution rate (scale bar) is 0.050, this value means that for every 100 bases, there are 5 different nucleotide substitutions. The existence of genetic distances affects the level of gene differences (genomic differences) in a population [28].
4. Conclusion
Molecular identification based on ITS rDNA sequences of K4 isolate that has been isolated from cherry fruits (M. calabura L.) has 96.30% similarity with taxa from the genus Candida. This shows the possibility that the K4 yeast isolate obtained was a new taxa closely related to D. rugosa (Syn. C. rugosa).

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