Molecular and phylogenetic analysis of inulinase-producing yeast isolated from nira siwalan (*Borassus flabellifer*) based on ITS sequences

D A Maryati, Wijanarka, R S Ferniah

Department of Biology, Faculty of Science and Mathematics, Diponegoro University, Jl. Prof H. Soedarto SH Tembalang Semarang, 50275, Indonesia

Corresponding author: wijanarka1810@gmail.com

Abstract. Inulinase (E.C. 3.2.1.7) is an enzyme capable of hydrolyzing inulin to be a fructose monomer, which is widely used in various fields, especially the food industry. This enzyme can be found in various plant species and can be produced by microorganisms such as bacteria, yeast, and fungi. Previous research has succeeded in isolating the potential yeast of inulinase from *nira siwalan* (*Borassus flabellifer*), named N1. This research purpose is to identify molecularly the yeast inulinolytic N1 and its kinship with other species based on internal transcribed spacer (ITS) rDNA sequences. The stages in this research are yeast DNA extraction using the chelex method, DNA amplification with primers ITS 4 and ITS 5, electrophoresis, sequencing analysis, and construction of the phylogenetic tree. Phylogenetic trees are reconstructed using the neighbor-joining method based on their evolutionary relationship. The results of DNA isolation showed that the concentration of yeast DNA was 495.8 ng/μl and purity of 2.12. The PCR product from the ITS fragment amplification produced a band size of ± 510 bp. The results of molecular identification and phylogenetic analysis showed that the N1 yeast isolate was closely related to *Candida parapsilosis* (MK63869.1) originating from India with a homology of 99.45%.

1. Introduction

Inulinase (E.C. 3.2.1.7) is an enzyme that capable of hydrolyzing inulin to fructose or fructo-oligosaccharides that work specifically on β-2,1 linkages of inulin [1]. Based on the mode of action in inulin, Inulinases are divided into endo- and exo-inulinase. Endoinulinase (β-2-1-D-fructan fructanohydrolase; EC 3.2.1.7) randomly cuts the internal links of inulin to release inulotriose, inulotetraose and inulopentaose as the main product. Exoinulinase (fructan β-fructosidase; EC 3.2.1.80) cuts terminal units from inulin sequentially producing fructose [2].

In recent years, inulinase has received increasing attention along with the increasing use of inulin in the industrial field. In industry, inulin is used as a substrate in the fermentation, food, pharmaceutical, biofuel, feed, and chemical industries [3]. Fructose monomers can also be used as a healthier and lower-calorie sweetener but sweeter than natural sugar (sucrose). Sucrose sweeteners cause many problems such as diabetes, carcinogenicity, atherosclerosis, and corpulence [4].

Inulinase can be produced from several plants, animals, and microorganisms such as bacteria, yeast, and fungi [1]. At present, the production of inulinase from microorganisms is more developed in the industry because of its rapid growth, genetic manipulation, and obtained in high quantities of enzymes [5]. Fungal strains are widely used because they can grow on inexpensive and stable substrates at high temperatures and low pH [6]. Inulinolytic yeast is a yeast that can produce inulinase [7]. Some types of
yeast that can produce inulinase are *Kluyveromyces marxianus*, *D. hansenii*, *Y. lipolytica*, *K. lactis*, *Pichia* sp., *Cryptococcus*, and several species of *Candida* sp. [8].

In a previous study, managed to isolate yeast from *nira siwalan* (*Borassus flabellifer*) which was given the code N1. The yeast isolate has the potential of inulinase with the enzyme activity of 0.469 (IU/mL). The morphological characteristics are the color of the white colony, the shape of a rounded colony with flat edges, oval cell shape, and has budding [9]. Based on these findings, it has not been reported that strains are truly superior so that this yeast application cannot yet be used on an industrial scale and further identification is needed.

Molecular identification in recent years has been widely applied for identification because the process is fast, sensitive, and accurate and can identify up to the level of species [10]. Molecular identification of organisms can use specific markers of inulinase genes or with universal yeast markers such as D1/ D2 sequences and internal transcribed spacers (ITS). ITS regions is commonly used for molecular identification of fungi [11]. This region is a non-coding region located in the ribosomal DNA (rDNA). The reason for using this marker is because the area is sustainable, the mutation rate is high so that it can be used to identify up to several species of strain strains, and is easily amplified because it has a high number of copies of genes [12] Therefore research is needed on molecular identification and phylogenetic analysis of yeast producing enzymes inulinase from *nira siwalan* (*Borassus flabellifer*) based on ITS sequences to determine specific yeast species, especially N1 isolates and their kinship with other species.

2. Materials and methods

2.1 Microorganisms

Yeast N1 is obtained from the isolation of *nira siwalan* (*Borassus flabellifer*) in Tuban, Indonesia [9]. The media used in maintaining culture stock and culture rejuvenation is inulinase selective medium (ISM). Media composition is 1 g pure inulin, 2 g agar, 1 g yeast extract in 100 mL distilled water [13].

2.2 DNA extraction

DNA isolation using the chelex method. Yeast N-1 aged 48 hours taken 3 ose was put in a 1.5 ml microtube containing ddH₂O 100 μl, then added 1 ml of phosphate buffer saline (PBS) and incubated at 4°C for 24 hours (overnight). The sample was centrifuged at 12,000 rpm for 10 min. Centrifugation aims to separate the solution into pellets and supernatants. The supernatant is removed using a micropipette. 1x PBS was added as much as 1 mL and centrifuged at 12,000 rpm for 5 min. The supernatant is removed, then ddH₂O is added as much as 100 μL and 50 μL 20% chelex solution. Samples were heated in a water bath for 95°C for 10 min, vortex every 5 min for 15-30 s. The sample was centrifuged at 12,000 rpm for 10 min. The supernatant was transferred to a clean microtube, the sample was stored at -20°C and amplified via PCR [14]. The concentration and purity of extracted DNA was measured with NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, USA) [15].

2.3 PCR amplification and electrophoresis

In this study, the ITS rDNA region was amplified using a primary pair: ITS 4 (5'- TCC TCC GCT TAT TGA TATGC-3') as reverse primer and ITS 5 (5'- GGA AGT AAA AGT CGT AAC AAG G-3') as a primary forward [16]. The total PCR mix is 25 μl consisting of 12.5 μl PCR kits (My Taq HS Red Mix), 7.5 μl uptrapure, 1.5 μl primers (10 pmol), and 2 μl (400 ng) DNA template. The Samples were amplified using a conventional T100 (Biorad) PCR with PCR conditions: initial denaturation at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 54°C for 15 s and extension at 72°C for 10 s. Post extension at 72°C for 5 min and holding at 4°C.

The PCR product was separated using 1% agarose gel electrophoresis in 0.5 mL TAE buffer 1 x, stained with fluorosafe as much as 4-6 μl. Gel migration was performed using an electroporator (Mupid-X) for 40 min at 100V in 1X TAE (Tris, EDTA Acetate buffer) [17]. DNA size can be estimated using 1 μl of
1Kb DNA ladder (Geneaid) and mixed with 1 μl loading dye as ballast. Electrophoresis results were visualized under UV light using gel documentation system (UVITEC).

2.4 Sequencing analysis
The results of PCR amplification are sequenced to find out the number and sequence of services through the services of PT. Genetika Science Indonesia. The base sequence reading method is the sanger method using the BigDye® Terminator Cycle Sequencing Kit v3.1.

2.5 Construction of the phylogenetic tree
The phylogenetic tree was constructed with Mega X and used the Neighbor-Joining method with a bootstrap analysis of 1000 replications [19, 20]. The Genetic distance was calculated using Kimura 2-parameter model (K2P), which was calculating base changes based on transitions and transversions [21].

3. Result and discussion

3.1 DNA extraction
Indicator of the quality of yeast DNA from extraction results is determined by the value of concentration and purity based on the value of the ratio λ260/ 280 [22]. The results of the DNA quality test for yeast N1 are shown in table 1. The yeast has good DNA concentration and DNA purity of more than 2.0 which indicates the presence of RNA contamination. DNA at a ratio of λ260/ 280 between 1.8-2.0 is pure DNA. Lower values indicate the presence of protein and phenol contaminants [23]. The above ratio indicates the presence of RNA contaminants [15].

Some things that affect the concentration and purity of DNA is when DNA isolation, especially in the last stage is not careful when taking supernatants so that material other than DNA is taken. RNA contaminated DNA can be purified by adding RNase. Ribonuclease (RNase) is an enzyme that can degrade RNA [24].

| Sample | DNA Concentration (ng/μl) | λ260 | λ280 | 260/280 | Sample Type |
|--------|--------------------------|------|------|---------|-------------|
| N1     | 495.8                     | 9.916| 4.667| 2.12    | DNA         |

3.2 PCR amplification and electrophoresis
PCR products were detected by 1% agarose gel electrophoresis showing a DNA band size of ± 500 bp (Figure 1). Based on the length of the band obtained, it shows that the ITS area can be well amplified. the size of the normal ITS amplification area for yeasts ranges from 300 bp - 900 bp depending on the species [25]. The success of gene amplification is influenced by temperature, especially the annealing temperature or the temperature of the primary attachment to the DNA template so that the DNA band is clear or does not smear. Annealing temperature that is too high causes the primer not to stick well to the template, while a low temperature will cause a non-specific band so that the amplified fragment is not desirable [17].
Figure 1. Electrophoresis DNA Amplification results of yeast N1 in 1% agarose gel with marker 1 kb

3.3 Sequencing analysis

Sequencing was used to determine the base sequence of the N1 yeast isolate. Sequence sequences are cut bases at the ends of 5' and 3' to get a good sequence of sequences and then merged between forward and reverse. The sequence of N1 sequences is compared with the ITS rDNA sequence available at GenBank to find its homology through searching BLAST at the National Biotechnology Information Center (NCBI). The alignment results of this isolate showed that yeast N1 had similarities with *Candida parapsilosis* MK638869.1 originating from India with an identity value of 99.45%. Yeast sequences compared to public databases are considered the same species or homologous if they have an identity value of 99% or higher [26]. The similarity lies in the simplicity of morphology (table 2).

| Characteristics     | Yeast N1 [9]          | C. parapsilosis [27]          |
|---------------------|------------------------|-------------------------------|
| Colony shape        | Round                  | Round                         |
| Colony color        | Yellowish white        | White to cream                |
| Surface texture     | Slippery, solid, hard  | Shiny and smooth              |
| Edge of the colony  | Align                  | Align                         |
| Cell shape          | oval                   | Oval                          |
| Asexual cells       | Budding                | Budding                       |

3.4 Phylogenetic analysis

Phylogenetic relationships were analyzed using the NJ (Neighbor-joining tree) method and tested with the Bootstrap method of 1,000 replications (Figure 2). Phylogenetic tree construction shows that the isolates of Yeast N1 are closely related to *C. parapsilosis* (MK638869.1) with a bootstrap value of 96%. Bootstrap values are declared stable if the value is above 95% and unstable or phylogenetic trees will change when reconstructed if the value is below 70% [28]. The genetic distance or scale bar in this study
is 0.05, which shows a change in base of 5 nucleotides every 100 base. The lower the bar scale value, the closer the kinship [29].

![Phylogenetic tree](image)

**Figure 2.** Phylogenetic tree of Yeast N1 constructed using Neighbor-Joining method based on sequences. *Saccharomyces cerevisiae* was used as an outgroup.

Yeast isolate N1 is an indigenous yeast isolate from nira siwalan as a producer of inulinase. *Candida tropicalis*, *Kloeckera japonica*, *K. apiculata*, *C. valida*, and *C. krusei* are yeast found in the nira siwalan (*Borassus flabellifer* L.) from Thailand [30]. *Saccharomyces cerevisiae* and *C. pararugosa* are yeast found in *Nira Borassus akeassii* from Burkina Faso, West Africa [31]. There are no studies that mention the yeast of *C. parapsilosis* isolated from nira siwalan. *C. parapsilosis* has a wide habitat in nature. *C. parapsilosis* is not only isolated from human skin and nails, but is also isolated from non-human sources such as domestic animals, insects, soil, and the marine environment [27]. However, based on previous research, yeast N1 which had been isolated from siwalan sap (Tuban, East Java) with 0.5 mM CaCl2 treatment had an inulinase activity of 0.468 IU / mL [9]. Yeast N1 can produce inulinase because in the silawan sap there is a relatively high sugar content of around 14.33%. Sugar is a carbon source in yeast in producing the enzyme inulinase [32].

Inulinase is an enzyme used to hydrolyze inulin. Commercial inulinase can be produced from microorganisms, one of which is yeast. Inulinase can be produced from several yeasts such as *Pichia* sp., *Kluveromyces marxianus*, *K. fragilis*, and *C. kefyr* [33]. In this study N1 yeast is known as *C. parapsilosis*, where *K. marxianus* teleomorphs from *C. parapsilosis* [34]. Yeast when grown will form structures for reproduction. Most yeasts reproduce asexually (anamorph) but along with the development of science, they find a sexual phase (teleomorph), and these anamorphic species will get a new name [35]. In this study, when yeast was carried out, molecular identification occurred in the phase anamorphic so that yeast does not produce the enzyme inulinase. Further research can be carried out when the yeast is experiencing a teleomorph phase and testing inulinase activity in several conditions to optimize inulinase production because the enzyme activity obtained in previous studies is still low.

4. **Conclusion**

In this study, yeast N1 isolated from nira siwalan (*Borassus flabellifer*) based on molecular identification and phylogenetic analysis was closely related to MK638869.1 *C. parapsilosis* originating from India with a homology value is 99.45%. This yeast is a potential producer of inulinase. Furthermore, it can be
continuous research can be carried out using yeast N1 during the teleomorph phase to test physiological characteristics and test the activity of inulinase enzymes in various conditions to optimize the production of inulinase before it is applied in industry, especially food and beverages.

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**References**

[1] Singh R S, Chauhan K and Kennedy J F 2017 *Int. J. Biol. Macromol.* 96 312–322
[2] Chen X, Xu X, Jin Z and Chen H 2013 *Carbohydr. Polym.* 92 1984–990
[3] Galindo-Leva L A, Hughes S R, López-Núñez J C, Jarodsky J M, Erickson A, Lindquist M R, Cox E J, Bischoff K M, Hoecker E C, Liu S, Qureshi N and Jones M A 2016 *J. Microbiol Biotechnol.* 43 927–939
[4] Jain S C, Jain P C and Kango N 2012 *J. Microbiol.* 43 62-69
[5] Singh R S and Singh R P 2010 *Food Technol. Biotechnol.* 48 435-450
[6] Singh R S, Singh T and Larroche C 2019 *Bior. Tech.* 273 641-653
[7] Wijanarka, Sutariningsih E, Dewi K dan Indrianto A 2012 *J. FKIP UNS* 609 – 611.
[8] Lachance M A, Boekhout T, Scorzetti G, Fell J W and Kurtzman C P 2011 *The Yeast Fifth Edition* (USA: Elsevier Press)
[9] Maisarah S, Wijanarka and Supriyadi A 2020 *NICHE J. of Trop. Biol.* 3 1-7
[10] Irinyi L, Serena C, Garcia-Hermoso D, Arbatazis M, Desnos-Ollivier M, Vu D, Cardinali G, Arthur I, Normand A-C, Giraldo A, et al. 2015 *Med Mycol.* 53 313–337
[11] Ferniah R S, Daryono B S, Kasiandari R S and Priyatmojo A 2014 *Microbiol Indones.* 8 121-126
[12] Edger P P, Tang M, Bird K A, Mayfield D R, Conant G, Mummenhoff K, Koch M A, Pires J C 2014 *Plos One* 9 1-7
[13] Ertan F, Aktac T, Kaboglu A C, Ekinci F, Bakar E 2003 *Pakistan J. Biol. Science.* 6 1386-1388
[14] Walsh P S, Metzger and Higuchi 2013 *BioTechniques* 54 134 – 139
[15] O’Neill M, McPartlin J, Arthur K, Riedel S and McMillan N D 2011 *J. Phys. Conf. Ser* 307 1-6
[16] White T J, Bruns T, Lee S and Taylor J 1990 *Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics* (New York: Academic Press Inc.) p. 315-322
[17] Sambrook J, Frisch E F and Maniatis T 2001 *Molecular Cloning, A Laboratory Manual. Edisi Ketiga* (New York: Cold Spring Harbour Lab. Press)
[18] Donkor E S, Nicholas T K, and Theophilus K A 2014 *J. Bioinform. Seq. Anal.* 6 2141-2464
[19] Kumar S, Stecher G, Li M, Knzyz C and Tamura K 2018 *Mol. Biol. Evol.* 35 1547-1549
[20] Saitou N and Nei M 1987 *Mol. Biol. Evol.* 4 406–425
[21] Kimura M 1980 *J.Mol. Evol.* 16 111-120
[22] Lucena-Aguilar G, Sánchez-López A M, Barberán-Aceituno C, Carrillo-Ávila J, Lopez Guerrero J A and Aguilar-Quesada R 2016 *Biop. Biob.* 14 264–270
[23] Isci B, Yıldırım H K, Altindisli A 2014 *J. Inst. Brew.* 120 238-243.
[24] Fatchiyah, Arumingtyas E L, Widyarti S dan Rahayu S 2011 *Biologi Molekuler Prinsip Dasar Analisis* (Jakarta: Erlangga)
[25] Pham T, Wimalasena T, Box W G, Koivuranta K, Storgårds E, Smart K A and Gibson B R 2011 *J. Inst. Brew.* 117 556–568
[26] Stamps J A, Yang L H, Morales V M and Mills K L B 2012 *Drosophila* regulate yeast density and increase yeast community similarity in a natural substrate *Plos One* 7 e42238
[27] Traofi D, Gacser A and Nosanchuk J D 2008 *Candida parapsilosis*, an Emerging Fungal Pathogen. *Clin Microbiol Rev*. **21** 606-625.

[28] Syaifudin M, Jubaedah D, Yonarta D and Hastuti Z 2019 DNA barcoding of snakeskin gourami *Trichogaster pectoralis* and blue bourami *Trichogaster trichopterus* based on cytochrome oxidase subunit I (COI) gene *IOP Conf Series: Earth and Environmental Science* **348** 1-6

[29] Rafidah N, Fitmawati, Juliartanti E and Sofiyanti N 2019 The infraspecies variations of *Mangifera foetida* based on the rbcl gene sequences. *J. Biol*. **12** 1-7

[30] Tuntiwongwanich S and Leenanon B 2009 Morphology and identification of yeast isolated from Toddy Palm in Thailand *J. Microsc. Soc. Thai.* **23** 34-37

[31] Ouoba L I, Kando C, Parkouda C, Sawadogo-Lingani H, Dawara B and Sutherland J P 2012 *J Appl Microbiol*. **113** 1428-1441

[32] Heryani H 2016 *Keutamaan Gula Aren dan Strategi Pengembangan Produk* (Banjarasin: Lambung Mangkurat University Press)

[33] Fernandes M R V S and Jiang B 2013 Fungal inulinases as potential enzymes for application in the food industry *J. Food Sci. Technol.* **5** 1031-1042

[34] Taei M, Chadeganipour M and Mohammadi R 2019 An alarming rise of non-*Candida albicans* species and uncommon yeasts in the clinical samples; a combination of various molecular techniques for identification of etiologic agents *BMC Research Notes* **12** 779

[35] Gandjar I, Sjamsuridzal W, Oetari A 2014 *Mikologi Dasar dan Terapan* (Jakarta: Yayasan Pustaka Obor Indonesia)