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Isolation, Identification, Screening, and Optimization of Endophytic Mold Fusarium sp in Red Dahlia (*Dahlia* sp.L) Tubers as Inulinase Producer

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ABSTRACT

Inulinase is an enzyme that converts inulin to fructose. This enzyme can be isolated from the endophytic mold of red dahlia tubers (*Dahlia* sp.L). The most critical steps to producing large-scale inulinase enzymes are to separate endophytic molds, then identify the type of mold and assess the best activity of the inulinase enzyme based on temperature and PH. The method is to isolate endophytic molds, make crude enzymes with Potato Dextrose Broth (PDB) media and fermentation culture, test the optimum PH of the inulinase enzyme, and conduct molecular identification of endophytic molds with ITS1 and ITS4 primers. The results obtained were the isolation of the red dahlia tuber mold, and five isolates were obtained, namely UD 1, UD 2, UD 3, UD 4, and UD 5. The results of qualitative inulinase screening by measuring the clear zone were UD 1:>2 (+ ++), UD 2:>2 (+++), UD 3: <1-2 (++), UD 4: < 1 (+), and UD 5: <1-2 (++). The result of the highest inulinase measurement at UD2 based on variations in temperature and PH was at a temperature variation of 350C and PH 6.0 was 1.322222222, and the lowest was at a temperature variation of 50 and PH 4.0 was 0.282716049. The result of identifying molecular based on BLAST analysis found Fusarium sp species, thus adding a new variant of the inulinase enzyme from red dahlia tubers that can be used by industry to produce fructose through enzymatic reactions.

1. Introduction

Inulinase is a hydrolytic enzyme that catalyzes the hydrolysis of polysaccharides into fructose or fructooligosaccharides and is widely used in the food and pharmaceutical industries. Many microorganisms, including molds, yeasts, actinomycetes, and other bacteria, are known to produce inulin hydrolyzing enzymes, however, the use of microbial enzymes and their biocatalyst applications needs much attention because they are still very limited, especially in the field of microbial biotechnology [1]. Inulin is a polysaccharide found in various plants including dahlia tubers. The utilization of dahlia tubers is currently

not optimal in the community and is considered as agricultural waste. Dahlia tubers are plants that contain carbohydrates and contain high levels of inulin. Inulin is very good as a dietary fiber, and used as a prebiotic. In addition, Inulin also has various physiological functions such as lowering blood sugar, blood lipids, anticancer, regulating intestinal microbial flora, and increasing absorption of minerals and vitamins [2]. Apart from dahlia tubers, inulin is found in chicory, Jerusalem artichoke, aguamie, asparagus, and agave [3]. Inulin can also be found in leeks, onions, garlic, wheat, and bananas [4].

Fructose is a natural low-calorie sugar, if in the same amount it will have a ratio of 1.5 to 2 times sweeter than conventional sugar. It is generally recognized as a safe food ingredient, so fructose is a sugar that is safe for use in diabetics [5]. Inulin produced from dahlia, chicory, and Jerusalem artichoke tubers mainly depends on the cultivation strategy, temperature, and harvest time of the plant. High content and degree of polymerization if the crop is harvested annually. Thus cost effectiveness, recency, and abundance in natural sources are the key factors that make inulin a potent substrate for producing Fructose and FOS [6]. Based on The Food and Drug Administration (FDA) it is agreed that inulin as a dietary fiber material is used to increase the nutritional value of food products. Due to its ability to gel, enhance sensory quality, and regulate texture, it makes inulin useful for applications as a functional ingredient in many foods. Because of the β-(2, 1) bond, inulin cannot be hydrolyzed by enzymes secreted by the human body and can only be fermented by bacteria in the large intestine, and inulin has dietary fiber and prebiotic functions [7].

The results of previous studies found that the endophytic mold Aspergillus tamarii-U4 has high activity at a temperature of 65^0C and PH 4.5 which has the potential for fructose production on inulin hydrolysis. Fusarium sp is an endophytic fungus that is pathogenic for several types of plants [8]. Fusarium sp is an endophytic mold that can be isolated from dahlia tubers and contains inulinase [9]. An important step that must be done to test the activity of the largest inulinase enzyme from Fusarium sp is to determine the optimum temperature and pH conditions. Temperatures ranging from 30, 35, 40, 45, 50^0C, and PH 4.0. 4.5. 5.0. 5.5. 6.0.

2. Materials And Methods

The design used in this study was descriptive observational by isolating endophytic molds from red dahlia tubers and then macroscopic, microscopic, and molecular identification, and screening for optimal inulinase activity based on temperature and pH. This research was conducted at the Integrated Laboratory of the Faculty of Medicine, Universitas Prima Indonesia and at the Macrogen Laboratory. The study was conducted in August 2022.

2.1 Endophytic Mold Isolation

Dahlia tuber material from North Sumatra as much as 300 grams was cleaned with running water for 5 minutes then sterilized by soaking in 70% alcohol for 5 minutes, followed by 1% NaOCL for 5 minutes then rinsed with distilled water for 5 minutes. After that it was cut into ± 2 cm cubes and put in a petri dish containing PDA media then incubated for 5 days and purified the mold as stock culture [10].

2.2 Macroscopic and Microscopic Observations

Macroscopic and microscopic observations were made by looking directly at the growth of molds, namely the shape, colony, color of the upper and lower surfaces and mycelium, while microscopic observations were carried out through staining of preparations using Lactophenol cotton blue (LCB) then examined under a microscope with 1000x magnification, carried out by identifying the color, spores, and hyphae [11].

2.3 Inulinase Activity Screening

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Examination Screening for inulinase activity by giving 3-5 drops of Lugol Iodine, let stand for 5 minutes while shaking so that it is even then cleaned with Aquadest then see the clear zone with the following conditions, colony area <1, given the symbol (+), moderate activity <1- 2 (++), high activity with ratio >2 (+++) [9].

2.4 Crude Enzyme Preparation

Fermented culture with 25 mL of fungal isolate dissolved in Potato Dextrose Broth (PDB) medium at pH 5.5. then incubated at a speed of 160 rpm and a temperature of 30ºC for 72 hours. Furthermore, extraction of crude enzyme inulinase as much as 2.5 mL of fermentation culture was put into an Erlenmeyer flask containing 25 mL of inulinase enzyme isolation media consisting of 1% inulin, (NH4)2PO4 0.5%, MgSO4. 7 H2O 0.05% and FeSO4 0.015 % pH 5.5. then incubated again for 120 hours at a speed of 160 rpm at 30^0C. Then centrifuged at 5000 rpm for 15 minutes. The filtrate is a crude inulinase enzyme [12].

2.5 Determination of the Fructose Calibration Curve

A standard fructose solution was prepared in several test tubes with a graded concentration of 20–90 mg/L from a fructose mother liquor of 1000 mg/L, then 1 mL of Nelson's reagent was added. Then the test tube was heated for 30 minutes and cooled again. After cooling, 1 mL of Arsenomolybdate solution was added, stirred until all the precipitate dissolved and 7 mL of distilled water was added, stirring again until homogeneous. Measure the absorbance with a UV-Vis spectrophotometer at a wavelength of 499 nm. So we get linear regression: y= 0.003x + 1.264 and R2= 0.998 [13].

2.6 Optimum Inulinase Activity Measurement

Testing of inulinase activity based on Optimum Temperature and PH, each test tube was filled with 1 mL of 0.1% inulin solution and 0.5 mL of crude enzyme. The test tube was added as much as 1 mL of 0.1 M acetate buffer at temperatures of 30, 35, 40, 45, 50^0C, and PH 4,0. 4,5. 5,0. 5,5. 6,0 and . The test tubes were incubated at 35ºC for 15 minutes [14]. 1 mL of Nelson's reagent was added. The test tube was heated on a water bath for 30 minutes. The test tube was cooled in running water to a temperature of 25ºC and 1 mL of Arsenomolybdate solution was added, stirring until all the precipitate was dissolved. Added 7 mL of distilled water, stirred again until homogeneous. Absorbance was measured by UV-Vis spectrophotometer at a wavelength of 499 nm [15].

2.7 Molecular Identification

The UD2 mold isolate was inoculated in 10 ml of PDB medium in a vial tube, then grown and in a rotary shaker for 72 hours, then centrifuged at 13000 rpm for 5 minutes, the supernatant was discarded and the pellet was removed, then the pellet was washed with 500 µL of TE buffer. (with pH 8) and 300 µL of extraction buffer, then added 150 µL of sodium acetate pH 5.2 and incubated at 20ºC for 10 minutes, then centrifuged at 13000 rpm for 5 minutes then the supernatant was taken as much as 600 µL and transferred into a new microtube then add Isopropanol with the same volume, then centrifugation at a speed of 13000 rpm for 10 minutes, after that wash the DNA with 500 µL ETOH (70% alcohol) after that dry the DNA and dissolve it with TE buffer as much as 20 l, then stored in the freezer at -20ºC [16].

The extracted DNA was then amplified using an Esco Swift MaxPro Thermal cycler. The ITS gene was amplified using ITS1 and ITS 4 Primers. The total volume of PCR was 50µL consisting of 2µL of forward primer; 2µL reverse primer; DNA template 4 L; GoTaq(R) Green (Promega) 25 µL; and 17µL Aquabides as solvent. The PCR was carried out under conditions of 1 initial denaturation cycle at 95oC for 5 minutes, 35 cycles consisting of 95oC denaturation for 1 minute, annealing 57.1oC for 1 minute, 72oC extension for 1 minute, final extension 72oC for 7 minutes, and storage temperature at final stage 4oC. The primers used

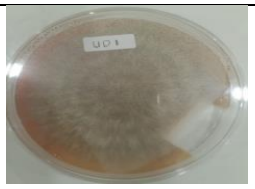
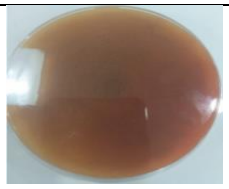
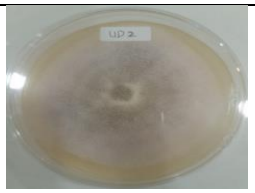
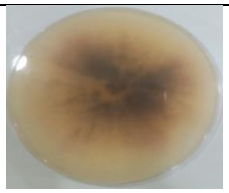
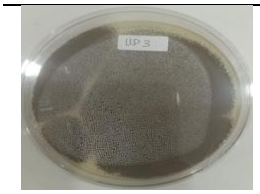
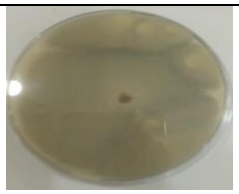
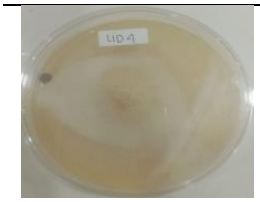
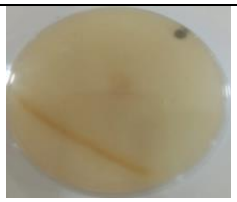
are ITS1/ITS4 universal primers, ITS 1 as reverse primers and ITS 4 as forward primers [17].

Analysis of the amplified DNA was carried out by electrophoresis using 0,8% agarose gel for DNA extraction, while for PCR amplification using 1,2% agarose gel. Electroporesis was carried out by taking 3 µL of marker 100 bp, and loading dye 1µL then resuspended and put into wells on 1,2% agarose gel, 3µL of PCR product from each sample was taken, then put into wells at 1,2 The % agarose gel was then electrophoresed for 30 minutes with a voltage of 110 volts. The finished agarose gel was then soaked with ethidium bromide for 15 minutes, then rinsed with sterile distilled water and analyzed with a UV transilluminator [18]. The amplified DNA was used for DNA sequencing steps based on the dideoxy nucleotide chain termination method (Macrogen Inc., South Korea). The results of DNA sequencing were then compiled using the Bioedit program and analyzed using the BLASTN program by utilizing information from *Genbank* (http://www.ncbi.nlm.nih.gov).

3. Findings And Discussion

3.1 Isolation of the Red Dahlia Tubers Endophytic Mold Macroscopically and Microscopically

The results of the isolation of endophytic molds of red dahlia tubers obtained 5 types of fungal isolates namely UD1, UD2, UD3, UD4, and UD5 which can be seen in Figure 1. macroscopic description and Figure 2. microscopic description as follows:

Isolate	Color (Surface and Referse)	Colony	Mycelium	Spore Color
 **UD 1. S**  **UD 1. R**	S: Yellowish-brown R: Reddish brown	Even and orderly	Cotton	Brown
 **UD 2. S**  **UD 2. R**	S: Purplish White R: The edges are brownish yellow and the middle is black	Even and orderly	Cotton	Purple
 **UD 3. S**  **UD 3. R**	S: Black R: Blackish Yellow	Even and orderly	Solid	Black
 **UD 4. S**  **UD 4. R**	S: White edges and yellow center R: Yellow	Even and orderly	Cotton	Yellow

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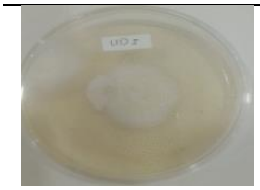
 UD 5. S	 UD 5. R	S: White R: White edges and yellow center	Even and orderly	Solid	White

Figure 1. Macroscopic Characteristics of Fungi

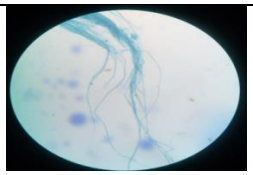
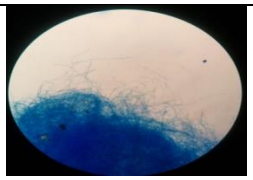
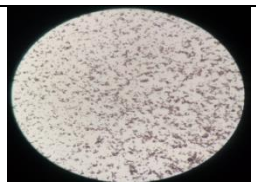
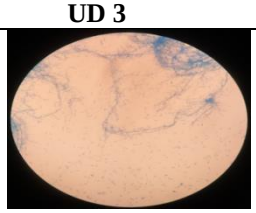
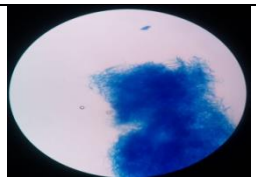
Isolate	Mycelium	Hyphae	Spore	Conidium
 UD 1	Unbranched mycelium and transparent stem	Hyphae septate	Spore (+)	Conidium round or globusa
 UD 2	Branched mycelium and transparent stem	Hyphae septate	Spore (+)	Conidium round or globusa
 UD 3	Mycelium and stems are not visible	Hyphae not visible	Spore (+)	Conidium round or globusa
 UD 4	Branched mycelium and transparent stem	Hyphae septate	Spore (+)	Conidium round or globusa
 UD 5	Branched mycelium and transparent stem	Hyphae septate	Spore (+)	Conidium round or globusa

Figure 2. Macroscopic Characteristics of Endophytic Molds

3.2 The Results of Inulinase Screening

The results of inulinase screening by measuring the area of the clear zone after the isolates were dripped with Lugol Iodine. Measurements were carried out with three repetitions. The results of Inulinase Screening can be seen in Table 1. below:

Table 1. Screening for inulinase activity

No	Types of Mold	Clear Zone Area	Information
1.	UD 1	>2	(+++):High Activity
2.	UD 2	>2	(+++):High Activity

3.	UD 3	<1-2	(++):Moderate Activity
4	UD 4	<1	(+):Low Activity
5.	UD 5	<1-2	(++):Moderate Activity

3.3 The Results of Measurement of Optimum Inulinase Activity Based on Temperature and PH

Testing of inulinase activity based on variations in temperature and PH is the Optimum Value of the inulinase enzyme at a temperature variation of 350 C and at PH 6.0 is 1.322222222 and the minimum value at a temperature variation of 500 C and at PH 4.0 is 0.282716049, overall it can be seen in Table 2 below:

Table 2. Measurement of Inulinase Enzyme Activity (U/ml)

Temperature and PH	Sample Absorbance	Fructose Concentration	Inulinase Enzyme Activity
Tem 30 PH 4,0	1,697	144,3333333	0,534567901
Tem 30 PH 4,5	2,095	277	1,025925926
Tem 30 PH 5,0	2,012	249,3333333	0,92345679
Tem 30 PH 5,5	1,996	244	0,903703704
Tem 30 PH 6,0	2,033	256,3333333	0,949382716
Tem 35 PH 4,0	1,523	86,33333333	0,319753086
Tem 35 PH 4,5	2,16	298,6666667	1,10617284
Tem 35 PH 5,0	2,025	253,6666667	0,939506173
Tem 35 PH 5,5	2,079	271,6666667	1,00617284
Tem 35 PH 6,0	2,335	357	1,322222222
Tem 40 PH 4,0	1,507	81	0,3
Tem 40 PH 4,5	1,988	241,3333333	0,89382716
Tem 40 PH 5,0	1,909	215	0,796296296
Tem 40 PH 5,5	1,603	113	0,418518519
Tem 40 PH 6,0	1,875	203,6666667	0,754320988
Tem 45 PH 4,0	1,5	78,66666667	0,291358025
Tem 45 PH 4,5	1,992	242,6666667	0,898765432
Tem 45 PH 5,0	2,11	282	1,044444444
Tem 45 PH 5,5	2,008	248	0,918518519
Tem 45 PH 6,0	1,989	241,6666667	0,895061728
Tem 50 PH 4,0	1,493	76,33333333	0,282716049
Tem 50 PH 4,5	2,142	292,6666667	1,083950617
Tem 50 PH 5,0	2,003	246,3333333	0,912345679
Tem 50 PH 5,5	2,105	280,3333333	1,038271605
Tem 50 PH 6,0	2,08	272	1,007407407

3.4 The Results of Molecular Identification

The Results of molecular identification of UD2 with ITS. The primers used for rDNA amplification and the results of DNA sequencing with ITS1 and ITS 4 can be seen in the Table 3. Figure 3, Figure 4, and Figure 5 following:

Table 3. Primers used for rDNA amplification in endophytic molds

Squencig Primer Name Primes Squences	PCR Primer Name Primer Squences
ITS1 5' (TCC GTA GGT GAA CCT GCG G) 3'	ITS1 5' (TCC GTA GGT GAA CCT GCG G) 3'

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ITS4 5' (TCC TCC GCT TAT TGA TAT GC) 3'	ITS4 5' (TCC TCC GCT TAT TGA TAT GC) 3'

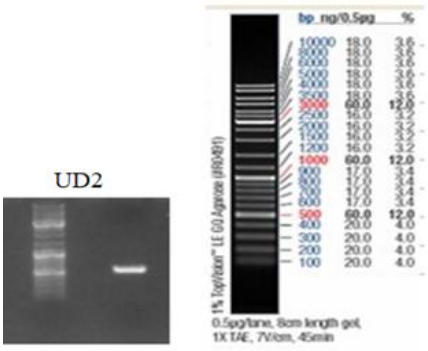

Figure 3. The results of DNA amplification of red dahlia tuber endophytic molds using ITS1/ITS 4 primer pair. M, 100 bp DNA ladder

DNA Squence of UD 2 ITS 1 abl 695

CCCGGTTTGGGGACAGGCGGGAGGGATCATTACCGAGTTTACAACTCCCAAACCCCTGTGAA
CATACCACTTGTTGCCTCGGCGGATCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGA
GGACCCCTAAACTCTGTTTCTATATGTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCA
ACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAA
TTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGG
GCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCACAGCTTGGTGTTGGGACTCGCGTTAAT
TCGCGTTCCTCAAATTGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCG
TTACTGGTAATCGTCGCGGCCACGCCGTTAAACCCCAACTTCTGAATGTTGACCTCGGATCAG
GTAGGAATACCCGCTGAACTTAAGCATATCAAACGGGGGGGGAAAAAAAGTCCTCTTCCCGCG
GAAGGCGGCCGGCGGGCCGTCGCGCGCGCCCGGACCGCCCCGCGGGGCCGGGGCACACG
ACGCCGCGGGAGCGGGCCGGGCACGCCCCCGGGCGAGCAGGACACAGGGGGATGGACAG
GAGGAGGGGAGA

DNA Squence of UD 2 ITS 4 abl 607

AGAACTTGGGTATTCTACTGATCCGAGGTCACATTCAGAAGTTGGGGTTTAACGGCGTGGCCG
CGACGATACCAGTAACGAGGGTTTTACTACTACGCTATGGAAGCTCGACGTGACCGCCAATCA
ATTTGAGGAACGCGAATTAACGCGAGTCCCAACACCAAGCTGTGCTTGAGGGTTGAAATGAC
GCTCGAACAGGCATGCCCGCCAGAATACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGA
TTCACTGAATTCTGCAATTCACATTACTTATCGCATTTTGCTGCGTTCTTCATCGATGCCAGAAC
CAAGAGATCCGTTGTTGAAAGTTTTGATTTATTTATGGTTTTACTCAGAAGTTACATATAGAAAC
AGAGTTTAGGGGTCCTCTGGCGGGCCGTCCCGTTTTACCGGGAGCGGGCTGATCCGCCGAG
GCAACAAGTGGTATGTTCACAGGGGTTTGGGAGTTGTAAACTCGGTAATGATCCCTCCGCTGG
TTCACCAACGGAGACCTTGTTACATTTTTTTTTTTCCAAACAGGTCTCGATAAACTCTCTGTCTT
TTTACACACCCCACCGGTCCACATCATCACTGTCATTT

Figure 4. UD 2 DNA sequence alignment verified by pairwise alignment using the Bioedit program

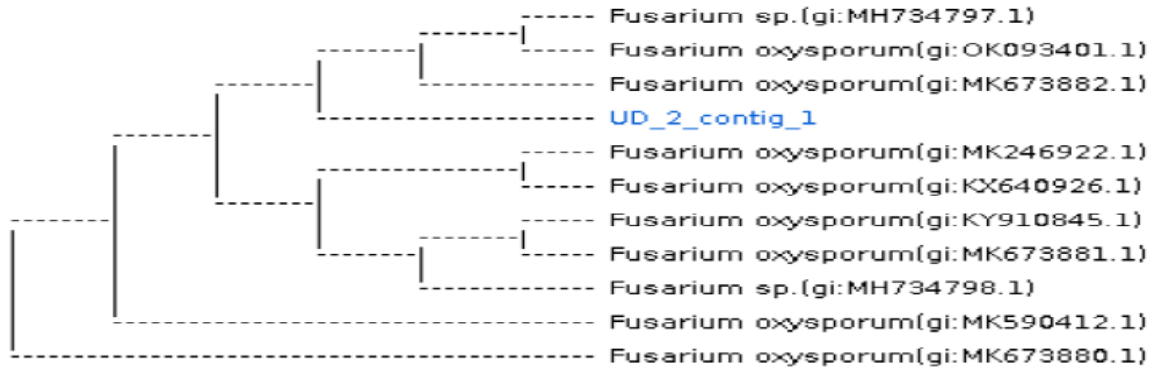

Figure 5. Neighbor-joining tree (NJ) with ITS sequence

The results obtained from molecular identification can be seen in Table 4. and Table 5. below:

Table 4. The identities of endophytic molds

Acession	Description	Length	Start	End	Coverage	Bit	E-Value	Macth/Total	Pct. (%)
MH734798.1	Fusarium sp	559	7	557	98	1013	0,0	550/551	99

Table 5. The strain of endophytic molds

Kingdom	Family	Genus	Species
Eukaryota	Nectriaceae	Fusarium	Fusarium sp.

Fructose and glucose are the main constituents formed by the hydrolysis of inulin using the enzyme inulinase. Inulin itself occurs in nature which is a polysaccharide mixture consisting of 20-30 units of fructose chains linked by β-(2,1) d-fructosyl-fructose bonds with a glucose molecule at the end of each fructose chain and is produced by some plants such as Jerusalem artichoke, chicory, dahlia and yacon [19]. Isolation of endophytic molds from red dahlia tubers containing high levels of inulin produced 5 types of endophytic molds, namely UD1, UD2, UD3, UD4, and UD5, with the highest inulin activity UD2.

Inulinase is an inducible enzyme and is important in industry and pharmaceuticals. This enzyme acts on the β-(2 → 1) bond in inulin, this enzymatic reaction can produce products such as fructose, glucose, and fructooligosaccharides. The most crucial initial step to having a high production is the selection of microbial strains that carry out enzymatic hydrolysis and biotransformation of inulin, as well as the accompanying fructo-polysaccharides [20].

In addition, the activity of the inulinase enzyme is influenced by temperature and pH. The results of the study obtained the highest inulinase activity at a temperature variation of 35^0 C and PH 6.0, namely 1.322222222 U/ml which was obtained from the isolation of the endophytic mold of Dahlia Merah tuber. The results of previous studies found that the highest test culture inulinase enzyme at PH 5 and a temperature of 45^0 C was obtained in the isolation of rotten garlic endophytic molds [21]. Another study also found that Aspergillus niger inulinase ATCC 20611 was optimal at pH 6.5, and a temperature of 30^0 C [22].

The results of the isolation of endophytic molds of red dahlia tuber UD 2 were carried out by molecular

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identification to obtain the species Fusarium sp. From the results of the study, there were differences in optimization based on variations in temperature and PH. Previous research stated that Fusarium sp based on the characterization of exo-inulinase and FOS production found that the optimum temperature and pH of the enzyme were found at 60^0C and pH 6.0, respectively. Meanwhile, the optimum inulinase production was at pH 5.0, and the incubation temperature was 30^0C for 120 hours. Under these conditions, the maximum inulinase concentration is 131.6 U/ml [23]. The results of previous studies found that Fusarium is a producer of inulinase which can also be isolated from red sunflowers, *Tithonia rotundifolia* [24].

Fructooligosaccharides (FOS) are linear oligomers of fructose units consisting of short chains of fructose molecules linked by β-2,1-glycosidic bonds so that they can be used as sweetening agents and bio preservatives in the food and pharmaceutical industries. This glycosidic bond cannot be hydrolyzed by human enzymes so it is safe for diabetics, can reduce triglyceride and cholesterol levels and increase the absorption of ions, such as Ca2+ and Mg2+ [25]. Fos is less digested in the digestive tract but FOS can maintain and stimulate the growth of probiotic bacteria such as bifidobacteria and lactobacilli [26]. The modulation of the gut microbiota can prevent the occurrence of NAFLD [27].

This research will add new variations of inulinase-producing endophytic mold species that can be used in the industrial world. Previously, many inulinase-producing microbes were found such as Yarrowia lipolitica, Cryptococcus aureus, Arthrobacter sp, Pseudomonas sp, Paenibacillus sp, Kluyveromyces sp. Y-85, and Aspergillus ficuum JNSP5-06 [28].

4. Conclusion

The results of the isolation of red dahlia tuber endophytic mold obtained five types of isolates labeled UD1, UD2, UD3, UD4, and UD5. From the five endophytic molds, macroscopic and microscopic observations were made. Based on the results of inulinase enzyme screening with the observation of the clear zone, it was found that the highest UD2 was >2 (+++) which had high activity. Furthermore, measurements of inulinase activity were carried out based on variations in temperature and PH, it was found that at a temperature variation of 35^0 C and at PH 6 it was 1.322222222 U/ml, and the lowest was at a temperature variation of 50^0 C and at PH 4 was 0.282716049 U/ml. Then the results of molecular identification on UD2 with primers ITS1 and ITS4 were found to be the species Fusarium sp.

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