Isolation and Characterization of Inulinase Producing Bacteria *Dahlia hybrida* Hort

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Abstract. The use of enzymes in the industrial world is growing, one of which is inulinase. This enzyme can hydrolyze inulin to fructose. The use of fructose as a food or beverage sweetener is more beneficial than sucrose. Inulinase can be produced by plants containing inulin and microorganisms. The ability of bacteria to produce inulinase is more effective and efficient because it is able to live in an extreme environment and is easily manipulated genetically. The purpose of this study was to isolate and determine the characteristics of inulinase-producing bacteria from the *Dahlia hybrida* Hort rhizosphere. This research was conducted using a descriptive method which was carried out in the Laboratory of Microbiology, Biotechnology, and Biochemistry, Faculty of Mathematics and Natural Sciences, UNP and the Biotechnology Laboratory of Andalas University. The working procedure is inulin extraction, bacterial isolation, morphological, microscopic and molecular observations which include the process of bacterial DNA isolation and amplification using the 16S rRNA gene. Data on bacterial characteristics were analyzed descriptively including morphology, microscopy, and molecular. The results obtained were four isolates of inulinase-producing bacteria which were classified as gram-negative bacteria with bacillary-shaped cells. However, it has a different colony morphology, namely LK1 isolate irregular shape and uneven colony edges, LK2 isolate round and flat colony edges, LK3 isolate in the form of roots and branched colony edges like fine threads and LK4 isolate round and flat colony edges. DNA isolation of inulinase-producing bacteria was successfully carried out, but amplification of the 16S rRNA gene was not successful. This is probably due to the not optimal annealing temperature in the PCR cycle.

1. Introduction
The enzyme industry has grown rapidly and occupies an important position in the industrial world. Increasing public concern for the environment and advances in environmentally friendly technology make enzyme technology an alternative to replace various chemical processes in the industrial sector. Enzymes are the catalysts of choice which are expected to reduce the impact of pollution and energy waste because their reactions do not require high energy, are specific, and are not toxic.

Enzymes play an important role in various food and non-food industries. The food industry, such as making fructose syrup (*High Fructose Syrup*), enzymes in fruit juice, tenderizing meat, peeling shrimp shells and shellfish contents, preventing changes in shrimp color, dissolving turbidity in tea color, and taste enzymes\(^[1]\). The role of enzymes in non-food industries such as the detergent industry, the leather.
industry, the dental poultice industry, and the pharmaceutical industry [2]. Inulinase is one of the enzymes that play a role in food processing [3].

Inulinase is a hydrolytic enzyme that catalyzes the hydrolysis reaction of inulin polysaccharides to fructose or fructo-oligosaccharides [4]. Inulinase can increase the production of fructose which is useful as a natural sweetener known as High Fructose Syrup (HFS) and can be used as an alternative to reduce dependence on cane sugar. HFS has a good market opportunity to be developed, but HFS production in Indonesia cannot be developed properly because there is no inulinase producer, the cost of importing enzymes is expensive, the enzyme produced is very small, and if the enzyme is hydrolyzed using acid at high temperatures it produces a color fraction that is dark and unwanted side-products such as difructofuran anhiera [5].

Efforts to overcome this problem need to find alternative sources of inulinase, one of which is microorganism biosynthesis. As a source of enzymes, microorganisms are more profitable because of their fast growth, can grow on cheap substrates, are easier to increase their yields through regulating growth conditions than plants and animals [6]. Microorganisms that are able to live at extreme temperatures to produce inulinase and are easily manipulated genetically are bacteria [7]. Bacteria are a group of microorganisms that are found in almost all places in both water, soil and air environments and are the most numerous compared to members of other microorganisms such as fungi, protozoa or actinomycetes [7].

*Dahlia hybrida* Hort. is a plant that contains inulin in its roots and tubers. To determine the potential bacteria to produce inulinase from the rhizosphere of *D. hybrida*, it is necessary to determine the morphological, microscopic, and molecular characteristics because bacteria are prokaryotic microorganisms that are difficult to observe directly by the eye.

Bacteria have special characters, are microscopic in size, and have a relatively simple structure. Apart from morphological and microscopic observations. Genetic approaches are also used to determine bacterial characteristics. The technique that is widely used is the ribosomal RNA technique, namely the 16S rRNA gene analysis. The use of the 16S rRNA gene as a target to determine the molecular characteristics of a bacterium can show the phylogenetic relationship of an individual based on its conservative and varied areas [8].

*Dahlia tubers* contain 70% inulin which can be used in the health sector. When inulin is hydrolyzed by certain enzymes or soil microbes, it turns into fructose, a sugar that is widely used in food preservation and the manufacture of fructose syrup. In principle, all types of dahlias contain inulin, but their levels and properties vary. Widowati *et al.* [9] reported that five types of dahlia tubers from the Cianjur area, West Java, had studied their inulin characteristics, found that the water content of fresh tubers ranged from 79.7% - 88.75%, while the inulin levels per 100 grams of dahlia tubers reached 65, 70%.

![Figure 1. Dahlia Plant Bulbs](image)

Inulin is a polysaccharide with the monomer fructose. Inulin is useful in the food sector, among others, as a substitute for fat and sugar in low-calorie food products and as a raw material for making fructose syrup. In the pharmaceutical field, inulin is used for kidney function tests [10]. Inulin is water-soluble, but cannot be digested by enzymes in the digestive system of mammals so that it reaches the
large intestine without undergoing structural changes. However, inulin can undergo fermentation due to microflora activity in the large intestine, which has positive implications for body health. According to, inulin is widely used by the food industry which produces snacks\[11\]. The structure of inulin is as follows:

![Figure 2. Structure of Inulin \[10\]](image)

Inulin contains carbohydrates with long chain sugars which are used to store energy in the roots or tubers of some plants that contain glucose and fructose, such as Jerusalem artichoke (*Helianthus tuberosus*), Chicorhi (*Chicoryum intybus* L), Dahlia (*Dahlia sp.* L), Yacon (*Smallanthus sanchifolius*) and Delion (*Taraxacum officinale*) and in small amounts are found in shallots, garlic, asparagus, bananas, and wheat. Abroad, more inulin is produced from Chicory tubers, while in Indonesia it is produced from Dahlia tubers\[10\].

Inulinase is a hydrolytic enzyme that catalyzes the hydrolysis reaction of inulin polysaccharides to fructose or fructooligosaccharides\[4\]. Inulin is hydrolyzed by inulinase to produce 90% fructose, therefore inulinase is a very potential commodity to be developed\[12\]. According to, the production of inulinase is higher in microbes, especially bacteria than inulin-producing plants because of their fast growth, easier to increase their yields through regulating growth conditions and easier for genetic manipulation\[2, 3\]. *Flavobacterium sp* are inulinase-producing bacteria that have been isolated from inulin-producing plants\[13\].

2. Materials and Methods

This type of research is descriptive research. Identification was carried out by morphological, microscopic and molecular observations to determine the type of inulinase-producing bacteria in the rhizosphere of *D. hybrida*. This research was conducted from November 2011 to May 2012 at the Laboratory of Microbiology, Biotechnology and Biochemistry, Faculty of Mathematics and Natural Sciences, Padang State University, as well as the Laboratory of Agricultural Biotechnology, Faculty of Agriculture, Andalas University.

2.1 Inulin Extraction

Inulin extraction from *D. hybrida* tubers was carried out by the following procedures: 1 kg of clean tubers were cut and mashed in a blender, added with distilled water in a ratio of 1: 2. The mixture was heated in a water bath at a temperature of 80°C - 90°C for 30 minutes. The mixture is still warm (above 50°C), filtered and the filtrate is taken. Furthermore, to the filtrate is added 30% ethanol as much as 40% of the volume of the filtrate. The solution is then stored in the freezer for 18 hours.

The solution was then left at room temperature for 2 hours and centrifuged at 1500 rpm for 15 minutes. The precipitate (wet inulin I) was added with distilled water (1: 2) then heated with a water bath at 70°C for 30 minutes. To this solution 0.56 g of activated carbon is added. The solution was
filtered with a vacuum, the volume measured and cooled at room temperature. Then the solution was centrifuged at 1500 rpm for 15 minutes until a white precipitate was obtained (wet inulin II). This precipitate is dried at a temperature of 50°C - 60°C for 6-7 hours, then the precipitate is mashed.

2.2 Isolation of Bacteria

Bacterial isolation was carried out by the procedure Castro [14] as follows: 1 g of soil sample was dissolved in 10 ml of 145 mM NaCl. The solution was heated at 37°C for 10 minutes. 5 ml of the suspension was added to the test tube containing 15 ml of liquid medium. Furthermore, the mixture was incubated using a 150 rpm shaker for 48 hours at 37°C. A total of 1.5 ml of the incubated mixture was added to 15 ml of the same medium (liquid medium). Then the mixture was incubated using a 150 rpm shaker for 24 hours at 37°C. Furthermore, as much as 0.5 ml of the mixture is put into the minimum 15 media. The mixture was incubated using a 150 rpm shaker at 37°C for 48 hours. The mixture was taken as much as 100 µl and put in an eppendorf tube containing 900 µl of distilled water. From this mixture a 10-1 dilution is obtained. Furthermore, a solution was made with a dilution of up to 10-5 by mixing 100 µl of the solution in the previous dilution with 900 µl of distilled water. Each solution at each dilution was inoculated on a solid medium on a petridish. This inoculation was carried out by taking 10 µl of each dilution by dropping it on the center of the solid medium. Furthermore, the surface of the medium is leveled with a grill glass so that the growth of bacteria is evenly distributed on all surfaces of the medium. Then incubated at 37°C for 48 hours. After 48 hours a bacterial colony will be formed which still consists of various types of bacteria. To get a pure bacterial colony consisting of only one type of bacteria, it can be done by re-inoculating the microbial colony on a new agar medium.

2.3 Bacterial Genomic DNA Isolation

The DNA isolation procedure is one of bacterial culture is put into 5 ml minimum media, then incubated using a shaker at a speed of 150 rpm at 37°C for 18 hours. Then 1.5 ml of inoculum from the mixture was put into the eppendorf and centrifuged at 10,000 rpm for 2 minutes and then the supernatant was discarded. Suspend the pellets in 500 µl of TE buffer by piping up down. Add 30-40 ml 10% SDS. Then add 3 µl 20 mg / ml proteinase K, the mixture is homogenized by turning the eppendorf slowly. Then incubated at 37°C for one hour. The next step, add 200 µl of phenol and 200 µl of chlorophore: isoamyl alcohol (24: 1). Then homogenize by turning the eppendorf slowly. Centrifuge at 10,000 rpm for 15 minutes at 20°C.

Take an aqueous layer and add sodium acetate (1/10 volume). Add 1 ml of absolute alcohol, then back and forth slowly. Store at -20°C for 24 hours. Centrifuge at 12,000 rpm for 30 minutes at -20°C. Then the supernatant is removed. The precipitate was washed using 70% alcohol by centrifuge at 15°C for 4 minutes at a speed of 12,000 rpm. Then the alcohol is removed and the DNA is dried and aerated with the eppendorf inverted position. After the DNA was dry, the DNA was dissolved in 20 µl ddH2O. The success of DNA isolation was confirmed by agarose gel electrophoresis method.

2.4 PCR reaction

PCR mix was made by inserting the isolated DNA into the PCR tube as much as 3 µl + 5.5 µl ddH2O + 12.5 µl Go Tag® Green Master Mix + 2 µl primer 27 F27F: 5’-AGAGTTGATCMTGGCTAG-3’ and + 2 µl 1525R primer: 5’-AAGGAGGTGWTCAREC-3’. The PCR tube which already contains the PCR mix is fed into the PCR machine.

3. Results and Discussion

3.1. Isolation of Inulinase Producing Bacteria in the rhizosphere of D. hybrida

Inulinase-producing bacterial isolates were obtained, each from the root and tuber rhizosphere. The types of bacteria found have different colony forms. Colony is a group of cells that are considered as
the progeny of one microorganism. Colony characteristics and inulinase-producing bacterial cells can be determined based on morphological and microscopic observations.

3.1.1. Characteristics Based on Morphology
Characteristics of bacteria that are determined based on morphology include size, shape, edge, elevation and colony color. Colonies were observed in bacteria that were 48 hours old. Colonies that produced inulinase were isolated from the root rhizosphere of *D. hybrida*.

**Bacteria Rizosfer Akar 1 (Bacteria LK1)**

![Figure 3. Bacteria Rizosfer Akar 1](image)

LK1 bacteria were isolated from the root rhizosphere of *D. hybrida*. It has a diameter of ≤ 1 mm, has an irregular shape (irregular), the edges of the colony are not flat (lobate), the elevation is raised and flat in the middle (raised) and has a greenish cream color that is thicker in the center and the edges of the colony look clearer.

**Bacteria Rizosfer Akar 2 (Bacteria LK2)**

![Figure 4. Bacteria Rizosfer Akar 2](image)

LK 2 bacteria were isolated from the root rhizosphere of *D. hybrida*. It has a diameter of ≤ 1 mm, has a round shape (circular), the edge of the colony is flat (Entire), hilly elevation (Umbonate) and has a reddish cream color that is darker in the center and the edges of the colony look clearer.

3.1.2. Microscopic Identification
The characteristics of bacterial cells can be observed microscopically. Cell shape and bacterial group were determined based on gram staining. The cells observed were 24 hours old because in old cultures many cell walls were damaged which resulted in the release of dye when washed with absolute alcohol, thus obscuring the results. The forms of inulinase-producing bacterial cells that were isolated from the root rhizosphere of *D. hybrida* are as follows:

**Root Rhizosphere 1 Bacteria (LK1 Bacteria)**

![Figure 5. Root Rhizosphere 1 Bacteria](image)

LK1 bacterial cells based on gram staining appear pink (pink) which indicates that the bacteria are gram negative. The shape of the LK1 bacterial cells is cylindrical or rods called bacilli. The ends of some cells look square and attached to each other so that they look like chains (Streptobasili)[16],[17].
LK2 bacterial cells based on gram stain look pink (pink) which indicates that the bacteria are gram negative. The cell shape of the LK2 bacteria is bacillus. The ends of some cells look round and there is an attachment between the two cells (Diplobasil)\textsuperscript{[16],}\textsuperscript{[17]}.

3.2. Inulinase Producing Bacteria Isolated From Tuber Rhizosphere

3.2.1. Identification Based on Morphology

Rhizosphere Bacteria Tuber 1 (Bacteria LK3)

LK3 bacteria were isolated from the rhizosphere of \textit{D. hybrida} root tubers. It has a diameter of $\leq 1$ mm, the shape is like a root (rhizoid), the edges of the colony are not flat like fine threads, the elevation is raised and flat in the center (raised) and has a cream color that is thicker in the middle and at the edges of the colony (the ends of the threads) looks clearer.

Rhizosphere Bacteria Tuber 2 (Bacteria LK4)

LK4 bacteria were isolated from the rhizosphere of \textit{D. hybrida} tubers. Has the same morphology as bacterial isolate 2, which is $\leq 1$ mm in diameter, circular in shape, the entire edge of the colony (Entire), hilly elevation (Umbonate) and has a reddish beige color with a darker center and clearer edges of the colony.

3.2.2. Microscopic Identification

Rhizosphere Bacteria Tuber 1 (Bacteria LK3)

LK3 bacterial cells based on gram stain look pink (pink) which indicates that the bacteria are gram negative. The shape of the LK3 bacterial cells is bacillus. The ends of some cells appear rounded and are more likely not to be attached to each other, so that they look like a single cell (monobasil)\textsuperscript{[16],}\textsuperscript{[17]}.
3.3. DNA Isolation and Molecular Characterization of Inulinase Producing Bacteria

Identification of inulinase-producing bacteria after morphological and microscopic observations, followed by molecular identification based on the 16S rRNA gene.

3.3.1. Isolation of Bacterial Genomic DNA

DNA isolation The genome of inulinase-producing bacteria starts from the destruction of cells until DNA is obtained. The success of DNA isolation can be proven after visualizing it using electrophoresis techniques. The results of the LK1 bacterial DNA electrophoresis can be seen in Figure 11.

From the figure 11, it can be seen that the isolation process of LK1 bacterial DNA was successfully carried out. This success was evidenced by the formation of a DNA band that has a length of 23,000 bp. It is stated that the length of the genomic DNA of a bacterium is approximately 23,000 bp. The genome is the DNA that makes up the chromosomes in the cell nucleus which can be used as a basis for data analysis, including to determine the molecular characteristics of bacteria.

3.3.2. Molecular Identification Based on the 16S rRNA gene

The isolated DNA from LK1 bacteria was used as a PCR template in the amplification of specific primary 16S rRNA genes, namely 27F and 1525R. Through this amplification process, it is hoped that a DNA band with a size of 1500 bp will be formed. It turned out that the amplification process did not get the desired length of DNA. This is known by analyzing PCR products by electrophoresis which can be seen in the figure.
Based on the Figure 12, it can be seen that the amplification process carried out on the DNA of LK1 inulinase-producing bacteria does not produce DNA bands at 1500 bp which indicates the presence of the 16S rRNA gene. This can be proven in Figure 12, the DNA band S column obtained is approximately the same length as the marker, which is 230 bp. The absence of the band is suspected due to several factors, including:

a. Annealing temperature is not optimal. Annealing is the process of attaching a primer to printed DNA\cite{18}.

b. The expected PCR product is too long (1500pb) so it requires appropriate time modification for each stage in the PCR cycle.

c. The isolated DNA is thought to still contain protein, so the quality is not good enough as a template.

The failure of the 16S rRNA gene amplification of LK1 inulinase-producing bacteria made molecular identification impossible. The use of the 16S rRNA gene as a target for molecular identification of a bacterium can show the phylogenetic relationship of an individual based on its conservative and varied areas\cite{8}.

4. Conclusion
1. Four isolates of inulinase-producing bacteria were obtained from the rhizosphere of D. hybrida.
2. Four isolates of inulinase-producing bacteria were classified as gram-negative with bacillary-shaped cells. However, they have different colony forms, namely LK1 bacteria are irregular in shape and uneven colony edges, LK2 bacteria are round and flat colony edges, LK3 bacteria are in the form of roots and branched colony edges such as fine threads and LK4 bacteria are round and flat colony edges. The molecular characteristics could not be determined because of the inadequacy of the 16S rRNA gene amplification process.

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