Utilization of dahlia tuber chips for preparation of difructose anhydride III (DFA III) by enzymatic reaction using inulin fructotransferase (IFTase)

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Abstract. Dahlia tuber is known as an alternative source of inulin. Inulin is a substrate required for difructose anhydride (DFA) III preparation by an enzymatic reaction. Therefore, dahlia tuber can be used as a raw material for the preparation of DFA III. Our previous investigation successfully produced DFA III from fresh dahlia tuber extract and inulin isolate from fresh dahlia tuber with a yield of 1.86 and 3.56 %, respectively. In this study, we aimed to compare the yield and purity of DFA III produced from dahlia tuber crude extract and inulin fraction prepared from dried slices (chips) of dahlia tuber. The dahlia tuber chips crude extract and inulin isolate were prepared by soaking the dahlia tuber chips in 70% - v/v ethanol at a ratio of 1:10. The soaked dahlia tuber chips were then further extracted and isolated to obtain the dahlia tuber chip crude extract and inulin isolate. The enzymatic reaction of DFA III was conducted at pH 5.5 and a temperature of 65 °C for 24 hours. The qualitative analysis of DFA III was carried out by thin layer chromatography (TLC). High-performance liquid chromatography (HPLC) coupled with a refractive index (RI) detector was conducted for quantitative analysis. The obtained syrup was decolorized and crystalized. The result showed that the extract gave a higher yield of DFA III (5.3 %) compared to inulin isolate (4.34 %). However, the extract showed a lower purity (93.4 %) compared to inulin isolate (94.35 %). This study confirmed the better potential of dahlia tuber chips than fresh dahlia tuber in producing DFA III.

1. Introduction
Dahlia plants are recognized by their beautiful flower. The flower is an important commodity of ornamental flowers industry, whereas the tubers are rarely used except for the plant’s reproduction [1]. *Dahlia pinnata* has edible root tubers that can be used in the human diet. Root tubers of dahlia contain inulin, a versatile natural reserve of polysaccharides [2]. Inulin is a polymer consisting of a chain of combined fructose monomer of which linked to each other in a straight chain through a bond β-(2-1) glycoside linkage with one terminal unit of glucose. It has functional properties as a soluble dietary fiber and widely used as a food ingredient. It is insoluble in cold water but dissolves in water at high temperature. Furthermore, inulin is an important substrate for the preparation of difructose anhydride (DFA) III by an enzymatic reaction. Therefore, dahlia tubers (Figure 1) become potential feedstock for the preparation of DFA III [3].
DFA III is a disaccharide cyclic compound. It consists of two residues of fructose bonded each other intramolecularly by dioxane bond. It is a non-reducing sugar with a melting point of 162 °C. DFA III is resistant to Maillard reaction. Additionally, the sweetness level of DFA III is half of the sucrose. DFA III is reported as functional food used for stimulation of calcium absorption [4]. Therefore, we suggest consuming DFA III as a dietary supplement [5]. DFA III can be prepared either chemically by pyrolysis reaction [6] or enzymatically by inulin fructotransferase (IFTase).

In our previous research, we successfully prepared DFA III from dahlia chips by an enzymatic reaction. The dahlia chips were reacted directly with IFTase in citric acid-NaOH buffer solution. The optimum result was obtained when the chips were blanched for 15 minutes in boiling water resulting in white and clear chips. The effective IFTase amount for DFA III preparation from 10 g of Dahlia tuber chips was 35 U [7]. In this present research, the dahlia tuber was processed into chips for the extraction and isolation processes prior to enzymatic reaction. Both the dahlia tuber chip crude extract and inulin isolate were used for DFA III preparation. This paper described the potential of dahlia tuber chips on the preparation of DFA III either using dahlia tuber chips extract or inulin fraction by an enzymatic reaction.

2. Materials and methods

2.1. Materials
Dahlia (Dahlia pinnata) tuber was purchased from a local farmer located in Lembang, Bandung, Indonesia. IFTase used for the preparation of DFA III was from Nonomuraea sp. Citric acid, sodium hydroxide, ethanol, sulfuric acid, n-butanol, 2-propanol, 4-methoxy benzaldehyde were of analytical grade from Merck, Germany.

2.2. Methods

2.2.1. Preparation of dahlia tuber chips. Fresh tubers were cleaned, peeled, and then blanched at 100 °C for 15 minutes. The blanched tubers were then sliced into pieces with an approximate width of 5 mm and dried in an oven blower at a temperature of 70 °C for not less than 8 hours (until dry) [7].

2.2.2. Preparation of dahlia chips extract. The preparation of dahlia tuber chips extract was carried out in accordance to Pudjiraharti et al [7] with a slight modification. The dahlia tuber chips (200 g) were soaked in 70 %-v/v ethanol (with a ratio of 1:10 w/v) for three days and the ethanol solution was replaced every 24 hours interval. The soaked chips were then separated from ethanol. Sequentially, the chips were blended and extracted with water (with a ratio of 1:15 w/v) at a temperature of 80-90 °C for 30 minutes. The extract was separated from the biomass by filtration.

2.2.3. Preparation of DFA III from dahlia tuber chips extract. About 1500 mL of Dahlia tuber chip extract was evaporated to a volume of 540 mL prior to enzymatic reaction. The enzyme reaction was performed in a 1 L glass reactor equipped with an electrical motor stirrer. The reaction mixture (600
mL) containing dahlia chips extract and 5 %-v/v IFTase in 10 mM of citrate-NaOH buffer pH 5.5 was incubated at 65 °C and stirred at 50 rpm for 24 hours. After incubation, the mixture was heated for 5 minutes in boiling water [3].

2.2.4. Isolation of inulin from dahlia tuber chips extract. The extracts obtained from 2.2.2 was isolated with 30 %-%-v/v ethanol (at a ratio of 5:2 for extract: ethanol) and allowed to stand at 0 °C overnight to precipitate inulin. The precipitated inulin was then separated directly by centrifuge at 7000 rpm for 10-15 minutes and dried in an oven blower at 50 °C for 18-24 hours. Inulin was then used for the preparation of DFA III by IFTase [8].

2.2.5. Preparation of DFA III from inulin isolate. Inulin solution (± 250 g/L) was mixed with 5 %-v/v IFTase in 10 mM citrate-NaOH buffer pH 5.5. The solution was then incubated at 65 °C, stirred at 50 rpm for 24 hours. After incubation, the mixture was heated for 5 minutes in boiling water [3].

2.2.6. Decolorization and crystallization DFA III. The syrups obtained from 2.2.3 and 2.2.5 were added each with 0.5 % w/v of commercial yeast and were stirred overnight at 30 °C. After the fermentation process, the liquids were filtered using no. 41 Whatman filter paper to separate the yeast. The fermentation liquid was then homogenized with activated carbon (1.5 %-w/v) for 30 minutes to remove the unexpected color of DFA III solution. Afterwards, the DFA III solution was subjected to vacuum filtration for separation of the activated carbon. The filtrate was then concentrated using a vacuum evaporator (50 °C) until 75-80 %-Brix. The concentrated liquid was then stirred at 50 rpm overnight to obtain DFA III crystal. The refined DFA III crystals were subsequently separated by vacuum filtration [8].

2.2.7. Analytical methods. Qualitative analysis of DFA III was performed by TLC method using silica gel 60 plate (Merck, Germany). The mobile phase for TLC was butanol:isopropanol:water (10:5:4, v/v). The DFA III spots were visualized by being sprayed with a reagent containing p-anisaldehyde:H₂SO₄:ethanol (1:1:18, v/v) and dried at 100 °C until maroon spots were formed. DFA III was measured quantitatively by HPLC instrument with a Refractive Index (RI) detector, equipped with an Aminex hpx-87p column. The mobile phase was water (100 %), the column temperature was 70 °C, and the flow rate was 1.0 mL/min [7].

3. Results and discussion

Fresh dahlia tubers can easily rotten at room temperature due to its high water content. In this study, prior to extraction and isolation of inulin, dahlia tubers were processed into chips in order to prolong its shelf-life as well as to prevent enzymatic degradation of inulin by indigenous inulinase. The dahlia tuber chips were soaked in ethanol 70 %-v/v to remove unwanted compound. As presented in Table 1, about 90-100 g of inulin isolate (6.4 %-yield w/w wet tubers) with a whiteness degree of 65-68 % were obtained from Dahlia tuber chips. Yakovsky and Kingsbury reported that solubility of inulin in alcohol is low [9]. However, during the soaking process of the dahlia tuber chips in 70 %-v/v alcohol, some of the inulin might have extracted out from the chips, thus resulting in a lower yield of inulin than that from fresh dahlia.

| Table 1. The water content, whiteness degree and yield of inulin from dahlia tubers. |
|-----------------------------------------------|-----------------|-----------------|-----------------|-----------------|
| **Tubers** | **Weight (g)** | **Inulin (g)** | **Water content of inulin (%-w/w)** | **Whiteness degree (%)** | **Yield (%-w/w fresh dahlia tubers)** |
| Fresh dahlia* | 1545.7 | 100.0 | 7.2 | 62.2 | 7.3 ± 1.2 |
| | 1545.7 | 127.0 | 7.7 | 62.4 | |
| Dahlia Chips | 200.0 | 90.0 | 9.4 | 65.0 | 6.4 ± 0.6 |
| | 200.0 | 100.6 | 9.4 | 68.2 | |

*Data from Budiwati et al [8]
Qualitative analysis of DFA III obtained from the enzymatic reaction was displayed on TLC chromatogram in Figure 2. It is obviously displayed that the longer time applied to the enzymatic reaction, the bigger and the thicker spots of DFA III were observed. At 6 hours of reaction time (H6), the inulin spots intensity was decreased as compared to initial reaction (H0) indicating the process of enzymatic reaction from inulin become DFA III. Inulin enzymatic reaction produced DFA III as the major compound and by-products saccharide including Sucrose (GF), Kestose (GF$_2$), Nystose (GF$_3$), and Fructosyl Nystose (GF$_4$) in a small quantity. As also shown in Figure 2A and 2B, the color intensity of GF$_2$ increased along with the reaction time suggested the conversion of GF$_4$ into DFA III and GF$_2$. Conversely, the intensity of the GF$_3$ spot decreased. It was probably due to GF$_3$ was converted into GF$_2$ and Fructose (F) by inulinase enzyme that might be present in the crude IFTase. The IFTase is reported breaking down the glycosidic bond of two adjacent fructose molecule. However, it does not break down the glycosidic bond between glucose and fructose in inulin [10]. We therefore suggested that GF$_2$ was further converted into GF and F. The existence of GF and F spots on lane H6, H12, H18 and H24 appeared in a very low intensity suggesting the inulinase enzyme has a very low activity [11].

The spots of inulin line in the lane of H6, H12, H18 and H24 on TLC chromatogram still appeared. Thus, showing the enzymatic reaction either for chip extract or inulin fraction for production of DFA III after 24 hours reaction was still continuing. Hence, the reaction time of 24 hours was not optimal for the enzymatic reaction of DFA III production. In addition, the presence of GF$_4$ may elevate the production of DFA III due to GF$_4$ was predicted as a substrate for DFA III production.

To remove the presence of by-products of saccharide other than DFA III, we conducted yeast fermentation. From the TLC chromatogram in the lane HF, it is obviously displayed that the spots of GF, GF$_2$, GF$_3$ and GF$_4$ disappeared, either in the reaction using chip extract or inulin fraction, yet not DFA III. Because DFA III was not metabolized by the yeast, we therefore suggested that yeast fermentation is a suitable refinement of DFA III after enzymatic reaction.

Table 2. Yield and purity of DFA III prepared from dahlia tuber extract.

| Tubers         | Weight (g) | DFA III (g) | Yield (g, fresh tuber) | Purity (%) |
|---------------|------------|-------------|------------------------|------------|
| Fresh dahlia  | 1545.7     | 30$^a$      | 1.9 ± 0.08             | 83.1 ± 0.9 |
|               | 1545.7     | 28$^a$      |                        |            |
| Dahlia Chips  | 200.0      | 76.1        | 5.3 ± 0.2              | 93.4 ± 0.1 |
|               | 200.0      | 81.5        |                        |            |

$^a$Data from Budiwati et al [8]
As seen in Table 2 and 3, dahlia tuber chips showed a higher yield of DFA III production than that from fresh Dahlia tuber. Study of Nordstrom and Swain reported the presence of various compounds from Dahlia plant including phenolic and flavonoid compounds [12]. We therefore suspected the presence of other compounds in fresh dahlia tubers that interfered with enzymatic reaction for DFA III production.

To confirm the purity of DFA III, we performed the HPLC analysis. The HPLC analysis was conducted on DFA III crystal. As also shown in Table 2, about 76-81.5 g of DFA III crystals were obtained from 200 g of Dahlia chips extract with a purity of 93.4 %. Whereas, the lower amount of DFA III was obtained from inulin isolate (65-70.9 g/200 gr dahlia chip) with a purity of 94.3 % (Table 3). When the purity of DFA III produced from inulin isolate was taken into account, it showed a similar result with DFA III produced from dahlia tuber chips extract. Thus, either dahlia tuber chips crude extract or inulin isolate can be used for the production of DFA III. However, due to the yield of DFA III production from dahlia tuber chips extract was three times higher than that of fresh dahlia tuber, we strongly suggested to utilize dahlia tuber chips extract for production of DFA III.

Taken together, all these findings confirm that processing of dahlia tubers to dahlia tuber chips elevates the production of DFA III.

4. Conclusions

In summary, this study showed that dahlia chip is more suitable for the production of DFA III than fresh dahlia tuber. In addition, DFA III can be produced either using chip extract or inulin isolate obtained from dahlia chip.

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Table 3. Yield and purity of DFA III prepared from inulin isolate.

| Tubers          | Weight (g) | DFA III (g) | Yield (%-w/w, fresh tuber) | Purity (%) |
|-----------------|------------|-------------|-----------------------------|------------|
| Fresh dahlia a  | 1545.7 a   | 49.5 a      | 3.6 ± 0.5                   | 90.3 ± 2.1 |
|                 | 1545.7 a   | 61.3 a      |                             |            |
| Dahlia Chips    | 200        | 65.2        | 4.6 ± 0.3                   | 94.3 ± 2.2 |
|                 | 200        | 70.9        |                             |            |

a Data from Budiwati et al [8]
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