Optimization of Medium Composition for Inulin Fructotransferase (IFTase) Production by *Nonomuracea* sp.

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Abstract. Inulin fructotransferase is an enzyme which converts inulin into difructose anhydride III (DFA III). Inulin fructotransferase was produced by fermentation in medium containing various concentrations of inulin (10 g/L, 20 g/L) as carbon source, yeast extract (1 g/L, 2g/L) as organic nitrogen source and (NH4)2SO4 (2g/L, 5g/L) as inorganic nitrogen source using *Nonomuracea* sp. ID06-A0189. The aim of the study was to optimize the medium composition using various concentrations of carbon and nitrogen sources for inulin fructotransferase production by *Nonomuracea* sp. ID06-A0189, through shake flask system. Fermentation was conducted at 30 °C, pH 7.0 and agitated in 130 rpm for three days. The result showed that comparable with those found in another condition the highest enzyme activity was produced in medium using inulin as carbon source with concentration of 10 g/L, yeast extract as organic nitrogen source with concentration of 5 g/L and ammonium sulfate as inorganic nitrogen source with concentration of 2 g/L. Addition of inulin as a carbon source increased the volume of biomass by about 11.5-24 mL. The enzyme activity in the culture supernatant reached 25.3 U/mL after three days incubation.

1. Introduction

Inulin fructotransferase (IFTase) is the liase group which can convert inulin to difructose anhydride III (DFA III) and a small amount of oligosaccharide. IFTase has been known were produced by genera and species of bacteria such as *Arhtobacter* sp.[1-3], *Basilus* sp.[4], *Leisofenia*[5], *Nonomuracea* sp. ID06-A0189[6,7], Streptomyces davawensis[8,9]. This study were performed by utilize *Nonomuracea* sp. bacteria ID06-A0189, that was isolated from Indonesian soils. This strain has potentiality to be used further in the development of IFT enzyme. *Nonomuracea* sp. ID06-A0189 IFTase has optimum activity at pH 5.5 and temperature of 65 °C. This enzyme is stable after heating 70 °C for 20 minutes without decreasing its enzyme activity but its activity found significantly decreased at 80 °C [6].

The amount of enzymes obtained is determined by the number of microbes in the medium, whereas the large amount of nutrition in the medium contained important nutrients source such as carbon,
nitrogen, minerals etc., were required to produce large number microbial population. Generally, the medium used for IFTase fermentation were known to be expensive, such as yeast extract (as a nitrogen source). In this study, ammonium sulphate were used as the nutrient for partial substitute the utilization of yeast extract. Previous study shows that the utilization of inulin mixture as carbon source, ammonium sulphate as inorganic nitrogen source and yeast extract as organic nitrogen sources has not provided optimal results for IFTase production. Otherwise, the medium using inulin as a carbon source and ammonium sulphate as inorganic nitrogen source were result in the highest enzyme activity (7.3 U/mL)[10]. The objective of this study is to find out the optimum condition of enzyme activity using mixture medium fermentation contained various concentration of inulin, various concentration yeast extract and ammonium sulphate. The results or this study are important for supporting the optimum production of DFA III production.

2. Materials and Methods

2.1 Materials

Medium and chemicals. Medium for the growth of Nonomuraea sp. ID06-A0189 was purchased from Difco, inulin with GR grade was the product of Orafti, Belgium, chemicals and organic solvents were from Merck.

2.2 Methods

2.2.1 Bacterial and culture conditions.

To maintain the bacteria, Nonomuraea sp. was grown on International Streptomyces Project 2 (ISP 2) medium which contains of 4g yeast extract, 4g glucose, 10g malt extract and 15g agar per liter (pH 7.3) and incubated at 30°C for 14 days.

2.2.2 Preparation inoculum.

Nonomuraea sp. ID 06-A0189 was inoculated into 50 mL of SR II medium in 500 mL shaking flask containing 1g soluble starch, 10g glucose, 3g beef extract, 5g yeast extract, 5g tryptone per liter (pH 7.0) and incubated at 30°C, agitation of 130 rpm for 2 days.

2.2.3 Production of IFTase and measurement of enzyme activity.

Inoculum of 5% (v/v) was inoculated into fermentation media containing various concentrations of inulin as carbon source, yeast extract as organic nitrogen source and (NH₄)₂SO₄ as inorganic nitrogen source as shown in Table 1, 2g NaNO₃, 0.5 g MgSO₄.7H₂O, 0.5g KCl, 0.01g FeSO₄.7H₂O, 0.5g KH₂PO₄, 2g CaCO₃ per liter (pH 7.0) and the inoculated media was incubated at 30°C, agitation of 130 rpm for 3 days. The culture was centrifuged to remove the biomass and the supernatant was used for IFTase activity determination.

| Composition of medium | Inulin (g/L) | NH₄SO₄ (g/L) | Yeast extract (g/L) |
|-----------------------|--------------|--------------|---------------------|
| 1                     | 10           | 2            | 1                   |
| 2                     | 10           | 2            | 2                   |
| 3                     | 10           | 5            | 1                   |
| 4                     | 10           | 5            | 2                   |
| 5                     | 20           | 2            | 1                   |
| 6                     | 20           | 2            | 2                   |
| 7                     | 20           | 5            | 1                   |
| 8                     | 20           | 5            | 2                   |
2.2.4 Analytical methods.
Inulin solution 10% (b/v) in 10 mM citrate buffer, pH 5.5 (0.5 ml), the enzyme solution (0.1 ml), water (0.4 ml) were mixed. Incubated in 65°C for 10 minutes, then boiled in 100°C, for 5 minutes to stop the reaction. The DFA III produced was determined by thin layer chromatography (TLC) using silica gel 60 plate (Merck, Germany) with a solvent system n-butanol: isopropanol:water (10:5:4, v/v). The plate was irrigated twice. The spots of reaction products were visualized by spraying the plate with a reagent containing p-anisolaldehyde:H₂SO₄:ethanol (1:1:18, v/v) and dried at 100°C until maroon spots were formed. The concentration of DFA III was measured by high-performance liquid chromatography (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. One unit of the enzyme activity was defined as the amount of enzyme which can produce 1 mmol of DFA III per min at pH 5.5 and 65°C [6]. Protein concentrations were determined by the method of Lowry, using bovine serum albumin as a standard [11].

2.2.5 Analysis inulin content.
Duplicate 1 ml filtrate samples were placed into test tube, added with 0.2 ml of 1.5 % systein, and 6 ml of 70% H₂SO₄. The mixtures were shaked and added with 0.2 ml of 0.12 % carbazol in ethanol solution, then heated at 60°C for 10 minutes. The sample then were stored in room temperature prior to absorbance measurement at at 560 nm wave length by spectrophotometer. A standard curve was prepared by using the standard which has inulin content 5 μg/mL -35 μg/mL [12].

3. Results and Discussion
The result of measurement pH medium after fermentation can be seen in Table 2. The pH value were affected by the presence of carbohydrate and protein that were produced from metabolism activity. Furthermore, the pH alteration during fermentation were influenced by the addition of ammonium sulfate. Excessive use of ammonium sulfate will reduce the pH of the medium thus allowing the fermentation conditions become too acidic. To prevent the change of pH in the medium, CaCO₃ was added to neutralize the organic acids produced during the fermentation, thus the pH of the medium can be maintained. This study indicated that the pH value were beyond the specific pH range for Nonomuraea sp. which is ranging from 5-10 that were proposed by Pudjiraharti et al (2011) [6], thus the pH value does not affected the bacteria growth.

Nonomuraea sp. growth can be observed by measuring the amount of biomass. The measurement of microorganism biomass in this study was conducted through placing the fermented culture in certain volume in a 50 ml centrifuge tube for 3 hours at 4 °C then measured its biomass volume. Microorganisms utilize nutrients in the medium for its growth and released metabolism product. This study indicated that the utilization of carbon and nitrogen sources and also protein production during fermentation were related to the cell growth. Table 2 shows that, increasing inulin as a carbon source in line with the increasing the volume of biomass. For instance, the addition of inulin 10 g/L produced 11.5-17.3 ml biomass, while the addition of inulin up to 20 g/L produced the volume of biomass up to 15.3-24 ml. Besides carbon, the increasing of Nonomuraea sp. growth also affected by nitrogen presence, for instance from Table 2 it can be seen that the addition of 1g/L yeast extract will enhance the volume of biomass up to 11.5-15.5 mL, furthermore with addition 2 g/L yeast extract the volume of biomass increases by 16.4-24 mL.

The addition of ammonium sulphate has play an important role for substitute the yeast extract as sources of inorganic nitrogen, but in this study the growth of Nonomuraea sp. seems were not affected by the addition of ammonium sulphate (see Table 2). The addition of ammonium sulfate 5 g/L to the medium contained with inulin 20 g/L possibly caused a stressful effect to the cell and lead to the suppress
synthesis of several enzymes that play a role in the metabolism process of the cell, thus inhibited the cell growth[13].

| Composition of medium | pH | Inulin consumption % | Biomass |
|-----------------------|----|-----------------------|---------|
| 1                     | 7.5| 78                    | 11.5    |
| 2                     | 6.5| 87                    | 16.5    |
| 3                     | 6.2| 79                    | 11.8    |
| 4                     | 6.6| 88.5                  | 17.3    |
| 5                     | 7.6| 56.5                  | 15.3    |
| 6                     | 7.6| 80.5                  | 24.0    |
| 7                     | 5.8| 52.8                  | 15.5    |
| 8                     | 6.1| 71.5                  | 18.8    |
| 9                     | 8.4| 36.0                  | 6.8     |

Consumption of inulin is a result of the differences between inulin used in medium with measurable residual inulin. Table 3 shows that the higher the biomass of Nonomuraea sp. volume observed, the higher the inulin consumed.

Enzyme activity test was carried out by reacting IFTase with the inulin substrate, where IFTase will convert inulin into DFA III and oligosaccharides[14]. The formed DFA III was analyzed qualitatively using the TLC method. The presence of enzyme activity can be seen from DFA III spots formed on the TLC plate.

**Figure 1.** TLC chromatogram of the result of enzyme activity measurement resulted in media with various concentration of inulin, ammonium sulphate and yeast extract.

Figure 1 shows TLC chromatogram for the reaction mixture of enzyme activity measurement for all media compositions. It can be seen that there were spots with the same Rf value as DFA III standard. This indicated the presence of IFT activity in the broth of all media composition. From DFA III spot intensity, it can be estimated that the higher the intensity of DFA III spot the higher the enzyme activity in the medium broth. Different intensity of DFA III spots were observed in different medium composition as seen in Figure 1. The highest intensity of DFA III spots were observed from medium 2 (inulin 10g/L, ammonium sulphate 2g/L and yeast extract 2g/L), medium 4 (inulin 10g/L, ammonium sulphate 5g/L and yeast extract 2g/L), medium 6 (inulin 20g/L, ammonium sulphate 2g/L and yeast extract 2g/L) and medium 8 (inulin 20g/L, ammonium sulphate 5g/L and yeast extract 2g/L). In addition, those media also showed high volume of biomass, therefore the samples of enzyme activity from those media were chosen for DFA III measurement quantitatively by HPLC method to calculate the value of enzyme activity.
Table 3. Enzyme activity

| Medium composition | Activity enzyme (U/mL) | Protein concentration (mg/mL) | Specific activity enzyme (U/mL) |
|--------------------|------------------------|------------------------------|-------------------------------|
| 2                  | 17.2                   | 0.4590                       | 37.5                          |
| 4                  | 25.3                   | 0.6317                       | 40.1                          |
| 6                  | 24.4                   | 0.6701                       | 36.4                          |
| 8                  | 23.4                   | 0.8992                       | 26.0                          |

Table 3 shows that the highest of enzyme activity of 25.3 U/mL. Compared to the result of TLC, the intensity of DFA III spots for medium 6 and 8 (20g/L inulin) were higher than that for medium 4 (10g/L inulin). In medium 6 and 8, some inulin in the medium has probably been converted to DFA III by IFT enzyme, therefore DFA III spot can be observed in the broth of media 6 and 8 (data not shown), thus the spots of DFA III of reaction mixture of enzyme activity measurement were thicker than that in medium 4. The activity of enzymes produced in liquid culture is influenced by the growth of microorganisms used and medium nutrient content. Poor quality of the inoculum culture result in less cell growth and low enzyme activity. Meanwhile, good quality inoculum causes abundant cell growth and high enzymes activity[15].

Proteins dissolved in fermentation medium need to be measured in order to determine the amount of enzyme proteins synthesized by microbes and to calculate enzyme specific activities. The concentration of protein in medium broth was determined by the Lowry method using Ciocalteu-Folin reagent to produce blue complex compounds measured at 500 nm. The results of protein measurement are shown in Table 3. Extracellular protein levels were measured to assess how far the increasing of activity was affected by the presence of other proteins which also dissolve in the enzyme extract. Futhermore, specific activity was examined by divided the enzyme activity with the enzyme protein content, by knowing the enzyme specific activity the amount of enzyme activity in proteins can also be determined.

4. Conclusions
The composition of medium is very necessary for Nonomuraea. sp growth. Nutrients are needed for microbial growth and enzyme production, one of which is carbon source and a nitrogen sources. The composition of medium produced the highest IFT enzyme activity of 25.3 U/mL used 10 g/L inulin as carbon sources, and ammonium sulphate of 5g/L and yeast extract of 2 g/L as inorganic and organic nitrogen sources respectively.

5. Acknowledgments
This work was supported by the Government of Indonesia through the DIPA project of the Indonesian Institute of Sciences.

6. References
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