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# Partial Purification and Characterization of Glucose Oxidase from *Aspergillus niger* IPBCC.08.610

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Glucose oxidase is an enzyme which catalyzes β-D-Glucose to gluconic acid and hydrogen peroxide. Glucose oxidase from *Aspergillus niger* IPBCC.08.610 was isolated, purified, and characterized. The enzyme was purified by ammonium sulphate precipitation and dialysis. The specific activity and yield of dialysis fraction were 19.766 U $mg^{-1}$ and 4.744%. The optimum pH and temperature were 6 and 30 °C. The stability of the enzyme at optimum pH and temperature decreased 50% at 25 minutes. The $k_m$ and $V_{max}$ values for enzyme were 27 Mm and 0.986 U $mg^{-1}$.

Key words: *Aspergillus niger* IPBCC.08.610, characterization, glucose oxidase, purification

Glukosa oksidase adalah enzim yang mengkatalis β-D-glukosa menjadi asam glukonat dan hidrogen peroksida. Glukosa oksidase dari *Aspergillus niger* IPBCC.08.610 diisolasi, dimurnikan, dan dikarakterisasi. Enzim dimurnikan dengan pengendapan amonium sulfat dan dialisis. Fraksi dialisis memiliki aktivitas spesifik 19,766 U $mg^{-1}$ dan menghasilkan 4,744%. pH dan suhu optimum adalah 6 dan 30 °C. Stabilitas pada pH dan suhu optimum menurun 50% pada menit ke-25. Nilai $k_m$ dan $V_{maks}$ enzim adalah 27 mM dan 0,986 U $mg^{-1}$.

Kata kunci: *Aspergillus niger* IPBCC.08.610, glukosa oksidase, karakterisasi, pemurnian

Glucose oxidase (EC 1.1.3.4) is an enzyme that catalyzed the oxidation of β-D-glucose using oxygen as an electron acceptor to produce hydrogen peroxide and gluconic acid (Singh and Verma 2010). Glucose oxidase (GOD) used as a food preservative, component of glucose sensor, and fuel cell applications (bio-fuel) (Bankar and Bule 2009). Biosensors using GOD could help the diabetic patient to monitor their blood sugar level. Glucose oxidase is a standard enzyme for biosensors. It had a relatively high-selectivity of glucose, easy to obtain, cheap, resistant to pH, ion, and temperature changes. These properties allowed unfavorable conditions in manufacturing and storage processes of the enzyme, especially for new users biosensors (Yoo and Lee 2010).

Naturally GOD produce by several fungal species with different characteristics and activities. *Penicillium amagasakiense (P. amagasakiense), Aspergillus niger (A. niger),* and *Penicillium notatum (P. notatum)* are fungi to produce GOD in industrial scale (Zia *et al.* 2007). *A. niger* is widely used as a major source of glucose oxidase commercially. Fiedurek *et al* (1986) explained that *Aspergillus niger* is the best source to produce glucose oxidase, because GOD from *A. niger* is more stable than GOD from *P. amagasakiense* (Holland *et al.* 2012). GOD of *A. niger* is an intracellular enzyme and it found in the mycelium of *A. niger* (Singh and Verma 2013). The mycelium of *A. niger* produce glucose oxidase that had a thermal stability in various mediums (Bhatti and Saleem 2009). The local isolate of *A. niger* IPBCC with code 08.610 which was isolated from *Dryobalanops* fence litter in Tarakan, North Kalimantan had several studies related to glucose oxidase production. Putri (2011) showed *A. niger* IPBCC 08610 could produce glucose oxidase as an intracellular enzyme. The optimum incubation time for GOD production was 48 hours (Triana 2013). The purification process by using ammonium sulfate precipitation method produced enzyme with activity 12.71 U $mL^{-1}$ (Rohmayanti *et al.* 2016).

Currently, the study related to the characterization of glucose oxidase very limited. Characterization of GOD is needed because the enzyme activity was influenced by several factors such as optimum pH, optimum temperature, enzyme kinetics, enzyme stability at pH and optimum temperature. Information regarding the enzyme characterization was required by the industry to improve the product quality and process efficiency. Therefore, this study aimed to purify and

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characterize glucose oxidase. The information regarding purification and characterization of glucose oxidase from *A. niger* IPBCC 08.610 could be utilized as an important reference source to produce an effective and efficient industrial glucose oxidase.

## MATERIALS AND METHODS

**Production and Isolation of GOD.** The media for glucose oxidase production contained 0.4 g $L^{-1}$ $(NH_4)_2HPO_4$, 0.2 g $L^{-1}$ $KH_2PO_4$, 0.2 g $L^{-1}$ $MgSO_4.7H_2O$, 40 g $L^{-1}$ $CaCO_3$, 3.3% sucrose, and 0.35% glucose. The medium was adjusted to pH 5.5 with the addition of 1 M $H_3PO_4$. The medium incubated at 200 rpm at 30 °C for 48 hours. After incubation, mycelium was separated from the production media by gauze. The filtered biomass was homogenized by using quartz sand and 0.1 M sodium phosphate buffer pH 6.0 and then it was centrifuged at 12000 rpm for 15 min. The supernatant was a crude extract of glucose oxidase. The protein concentration and enzyme activity of crude extracts were analyzed. Afterward, the crude extract was purified.

**Determination of Protein Concentration and Enzyme Activity.** For determination of protein concentration, 20 µL samples were added by 1000 µL of Bradford reagents, incubated for 10 min, and measured the absorbance at 595 nm. The standard curve was made by using Bovine Serum Albumin (BSA) solution with concentration around 0.1-0.5 mg $mL^{-1}$. The linear regression equation of standard curve was used to determine the protein concentration the sample. Glucose oxidase activity was the amount of glucose oxidase used to catalyze 1 µmol β-D-glucose to $H_2O_2$ per minute. To determine the enzyme activity, 170 µL 10% glucose (w/v), 800 µL o- dianicidine (6.6 mg o-dianicidine (100 mL buffer potassium phosphate 0.1 M pH 7), 30 µL horseradish peroxidase (HRP) 60 U $mL^{-1}$, and 30 µL GOD were mixed. The oxidation reaction of o-dianicidine was analyzed by measuring the absorbance at 436 nm.

**Purification of Glucose Oxidase**. Crude extract was mixed and homogenized with 80% saturated ammonium sulfate for 1.5 hours at 4 °C, and centrifuged at 12000 rpm for 15 minutes. The precipitate fraction was separated and dissolved in phosphate buffer 0.1 M pH 6, and purified by using dialysis process. Dialysis process used molecular weight cut off 12 kDa. The fraction was put into the dialysis tube and dialyzed for 9 hours in 0.001 M phosphate buffer pH 6 solution. The buffer solution was replaced every 3 hours. The dialysis process was stopped when the enzyme fraction did not produce a white precipitate if $BaCl_2$ was added into the buffer solution.

**Determination of Optimum pH and Temperature**. Phosphate citrate buffer with concentration 0.1M pH 4 to 7 with interval 0.5 were used to determine the optimum pH. The buffer from assay determination was replaced by the test buffer, and the absorbance was measured at 436 nm. For optimum temperature determination, GOD assay reagents were incubated for 10 min at some temperature conditions (25, 30, 35, and 40 °C) and the absorbance was measured at room temperature at 436 nm.

**Enzyme Stability at Optimum pH and Temperature**. The thermal stability of GOD was determined by incubating the reagent enzyme assay at optimum temperature and pH for 10, 20, 40, 50, and 60 minutes. The enzyme activity of the treatments was compared with pre-incubation activity. The stability of the enzyme was expressed as a percentage of residual activity and it was calculated by using the following equation:

$$\text{Residual activity}(\%) = \frac{\text{enzyme activity after incubation}}{\text{enzyme activity before incubation}} \times 100\%$$

**Kinetics of Glucose Oxidase.** Determination of enzyme kinetics by measuring the enzyme activity at several substrate concentrations. The substrate solution was glucose with concentration range 10-100 mM. The result obtained was applied into the Michaelis-Menten curve. Through the Michaelis-Menten curve, a range of substrate concentrations which showed a significant increase in enzyme activity was selected to create Lineweaver-Burk plot and determine $k_m$ and $V_{max}$.

## RESULTS

**Production and Isolation of GOD.** Glucose oxidase was produced by growing the mycelium of *A. niger*. The mycelium had white color and round shape. Quartz sand was added to the mycelium to take glucose oxidase from the cell. After mycelium was homogenized by using quartz sand, the sample was centrifuged. Pellets and supernatant were separated after the centrifugation process. The supernatant was a crude extract of glucose oxidase.

**Purification of Glucose Oxidase**. The purification results showed an increase in the purity of glucose

oxidase. The specific activity of crude extract was 0.248 U $mg^{-1}$. The results showed that the activity of glucose oxidase from ammonium sulfate precipitation method was 1.168 U $mg^{-1}$, and it was increasing 4,709 times if it compared to the activity of crude extract. The protein concentration of ammonium sulfate fraction was 6.84 mg, and it was decreasing if it compared to the protein content of crude extract. The ammonium sulfate fraction was purified by dialysis process. The dialysis process showed a significant increase in the purity of the sample was 79.701 times bigger than the initial (Table 1).

**Optimum pH and Temperature.** The pH and the optimum temperature were determined at the highest activity. The activity of glucose oxidase was increasing at pH 4 to pH 6. The highest activity was 1.438 U $mL^{-1}$at pH 6 of (Fig 1). At pH 6.5 and 7, the activity was decreasing by 72% of the highest activity. At temperature 30 °C, the activity was increasing, and it was starting to decrease as the temperature was higher than 30 °C. The activity at temperature 30 °C was increasing 282% than the activity at 25 °C, start from 1.062 U $mL^{-1}$ and increased into 2.992 U $mL^{-1}$. At temperatures of 35 °C and 40 °C, the activities were decreasing, respectively 1.477 U $mL^{-1}$and 1.168 U $mL^{-1}$ (Fig 1).

**Stability at Optimum pH and Temperature.** Figure 2 shows the residual activity at optimum pH and temperature. The residual activity was decreasing at treatment time was increasing. In 25 minutes of treatment time, the residual activity was decreasing 50% of the initial. In 60 minutes of treatment time, residual activity was decreasing 37.5% of the initial.

**Kinetic Glucose Oxidase.** The Michelis-Mentens curve showed the rate increase until 0.03 M glucose concentration. The Michelis-Mentens curve plot in the range 0.01 to 0.03 M of substrate concentration was transformed into the Linewave-Burk curve. Through the Lineweaver-Burk curve, $k_m$ and $V_{max}$ values were obtained, respectively 27 mM and 0.986 U $mg^{-1}$ (Fig 3).

## DISCUSSION

**Production and Isolation of Glucose Oxidase**. *Aspergillus niger* was a fungus with the genus *Aspergillus*, and the spores were black or dark brown (Gautam *et al*. 2011). The production of glucose oxidase from *Aspergillus niger* IPBCC 08.610 used a liquid medium contain $(NH_4)_2HPO_4$, $KH_2PO_4$, $MgSO_4.7H_2O$, $CaCO_3$, sucrose, and glucose. $(NH_4)_2HPO_4$served as a source of nitrogen and medium of pH control (Khursid *et al*. 2011). The concentration of $MgSO_4.7H_2O$ used to be very low, because excess $Mg^{2+}$ ions would decrease GOD production (Hamid *et al.* 2003). Sucrose and glucose were used as carbon sources that could affect the formation of biomass mycelium and cell metabolites. The concentration of sucrose was higher than glucose, because sucrose was a substrate that could produce GOD more optimal. The excessive glucose concentration could affect the pH culture, it could decrease the mass of mycelium, and the concentration of GOD produced (Bankar and Bule 2009). $CaCO_3$ was added to induce GOD formation due to a shift of metabolism from glycolysis to the phosphate pentose pathway. $CaCO_3$ could inhibit the synthesis of 6-phosphofructokinase and the concentration of glucose-6-phosphate dehydrogenase was increasing, so the production of GOD was also increasing (Moorthi 2009). Environmental pH might affect to the nutrient solubility, enzyme synthesis rate, and reduction oxidation reactions (Bankar and Bule. 2009). The optimum pH for the production of GOD was 5.5 (Singh and Verma 2010). The types of isolates, medium of nutrients, and physiological conditions, the

Table 1 Purification table from intracellular glucose oxidase from *A. niger* IPBCC 08.610

| Fraction | Activity (U $mL^{-1}$) | [Protein] (mg $mL^{-1}$) | Volume (mL) | Total activity (U) | Total Protein (mg) | Specific activity (U $mg^{-1}$) | Yield (%) | Increasing ratio, compared to crude extract sample |
|---|---|---|---|---|---|---|---|---|
| Crude extract | 0.617 | 2.445 | 50 | 30.838 | 122.25 | 0.248 | 100 | 1 |
| Ammonium sulfate | 1.998 | 1.71 | 4 | 7.992 | 6.84 | 1.168 | 25.916 | 4.709 |
| Dialysis | 2.151 | 0.109 | 0.68 | 1.463 | 0.074 | 19.766 | 4.744 | 79.701 |

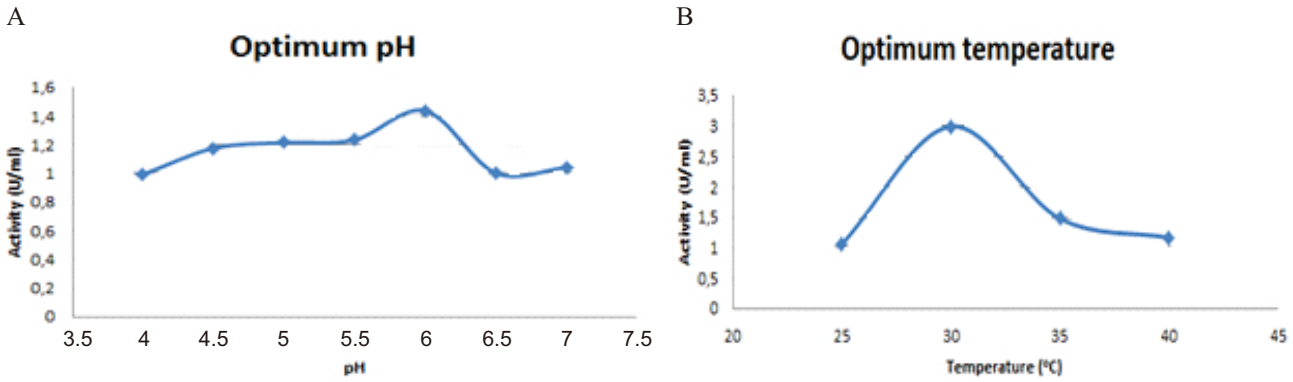


Fig 1 Determination of (a) Optimum pH and (b) Optimum temperature.

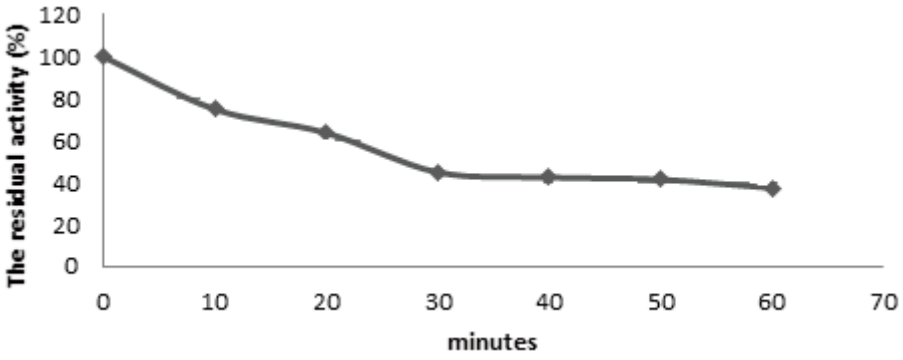


Fig 2 Stability at optimum pH and temperature.

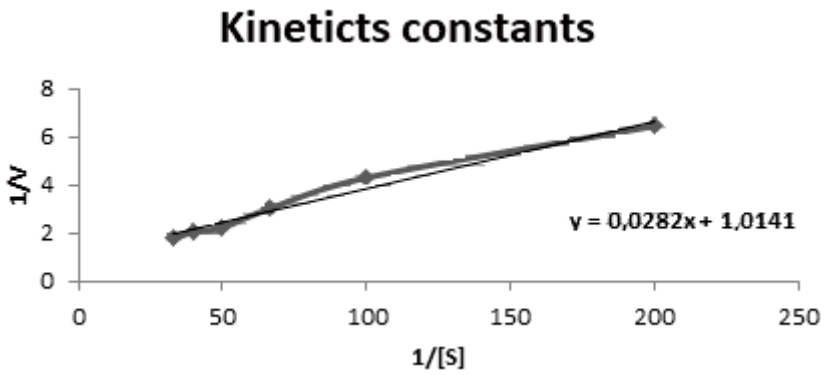


Fig 3 The Lineweaver-Burk curve.

incubation time was also affecting the production of glucose oxidase (Sabir *et al.* 2007). According to Triana (2013), the GOD activity of *A. niger* IPBCC 08.610 was reaching maximum after 48 hours of incubation. It was indicated by the GOD activity 48 hours was higher than 72 hours of incubation. The enzyme activity decreased due to the accumulation of the hydrolysis product (Purkan *et al.* 2014).

Glucose oxidase was produced from *Aspergillus niger* metabolites (Haq *et al*. 2013). *Aspergillus niger* IPBCC 08.610 produced glucose oxidase as an intracellular enzyme (Putri 2012). It was indicated by the enzyme activity of intracellular enzymes was higher than the extracellular enzymes. Glucose oxidase as an intracellular enzyme was presented in the mycelium (Bhat *et al*. 2013). Glucose oxidase was obtained by the breakdown of the *A. niger* mycelium and the cell membranes, so that enzymes could be extracted and obtained as the crude extracts. Crude extract of glucose oxidase was purified by ammonium sulfate precipitation.

**Purification of Glucose Oxidase.** Crude extract GOD was purified by using two steps of purification processes, they were ammonium sulfate precipitation and dialysis process. The crude extract still contained carbohydrates, lipid and protein. So, it needed a

purification process to get the enzyme with appropriate activity (Wahidah *et al*. 2017). The crude extract was purified by ammonium sulfate precipitation method. Ammonium sulfate precipitation was the purification method based on the interactions between water, proteins, and salts. At certain saturation levels, the water molecules bonded to the salt, so the proteins would be settled down (Abelson *et al.* 2014). The saturation level of Ammonium sulfate for glucose oxidase purification was optimized first. Ammonium sulfate with 80% of saturation level produced the highest glucose oxidase activity (Triana 2013). The ammonium sulfate with 80% of saturation level could be interpreted that the protein was more hydrophilic (Setiawan *et al*. 2013). The fraction of the ammonium sulfate was purified by dialysis process. Dialysis process was performed to remove the ammonium sulfate in extract, so the fraction only containing proteins (Nelson and Cox 2013). The membrane size used was 12 kDa, so the molecules with size less than 12 kDa could be removed and the desired protein would be remained inside the membrane (Sattayasai 2012).

**Optimum pH and Temperature.** Temperature and pH could affect the enzyme activity. pH and temperature could damage the structure of the enzyme. If the protein structure was changed, then the active side of the enzyme would be disturbed. The changed active side could decrease the enzyme ability to bind with the substrate (Voet and Voet 2010). pH and temperature that produced the maximum enzyme activity were called the optimum pH and temperature. Enzyme activity at the optimum pH and temperature reaches the maximum value because amino acids on the active side are at the appropriate conformation to bind to the substrate. So it could bind optimally with the substrate. The activity at pH lower and higher than the optimum condition were decreasing due to pH could affect the ionic bonds and hydrogen bonds on the catalytic side (Reece *et al*. 2010).

**Stability at Optimum pH and Temperature.** The GOD from *A. niger* IPBCC 08.610 had optimum activity at pH 6 and 30 °C. The information of stability at optimum pH and temperature were used to determine the decrease of activity before the enzyme was becoming inactive. The glucose oxidase was not active effectively at 30 °C without stabilization. The immobilization process could stabilize GOD from *A. niger* (Simpson *et al*. 2007). The most of protein molecule were denatured at high temperature and long exposure of heating time. The denaturation of enzymes accompanied by disruption hydrophobic interactions. That was effect by the dissociation subunit, so the enzyme activity was decreasing (Singh and Verma 2013).

**Kinetic constants.** The rate of hydrolysis or the decomposition reaction was called velocity (V). The speed of the reaction was increasing with the increasing of substrate concentration. The speed in linear correlation was called the maximum speed ($V_{max}$). The km value indicated that a half of the enzyme active side was binding to the substrate (Nelson and Cox 2013).

The $V_{max}$ and $k_m$ values could be affected by enzyme amount, purity level, and environmental conditions, such as pH and temperature. The low $V_{max}$ value was obtained due to enzyme concentration was too low, and the formation of the enzyme substrate complex was limited, so the reaction rate was decreasing (Iswantini *et al*. 2009). The low $k_m$ value indicated that the glucose oxidase had a high affinity to the substrate, so the bond of the enzyme and substrate was getting stronger and causing the equilibrium of the reaction toward the formation of enzyme substrate complex (Campbell *et al*. 2004).

To conclude, glucose oxidase was isolated from mycelium *A. niger* IPBCC 08.610 as intracellular enzyme. GOD was purified by ammonium sulfate precipitation method and dialysis process with enzyme activity and yield respectively 19.77 U $mg^{-1}$ and 4.74%. The optimum pH and temperature were 6 and 30 °C. At the optimum pH and temperature showed the stability of enzyme was decreasing 50% at 25 minutes. The $k_m$ and $v_{max}$ values for the enzyme were 27 mM and 0.986 U $mg^{-1}$.

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