D-Amino Acid Oxidase Production from Cassava Glucose Syrup by *Trigonopsis variabilis*

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Abstract— Three alternative carbon sources (molasses, cassava glucose syrup and sorghum fructose syrup) on the production of D-amino acid oxidase (DAAO) from Trigonopsis variabilis (TvDAAO) has been studied.The aim of this study was to screen out the best alternative carbon sources that can be utilized in TvDAAO production, and to study the effect of the additional nitrogen sources. Screening of carbon sources were carried out using glucose, molasses, cassava glucose syrup (CGS) and sorghum fructose syrup (SFS), at the same glucose levels. Analysis of TvDAAO was measured by o-phenylenediamine/horseradish peroxidase coupling assay. The best alternative carbon source was selected for optimization at various concentrations. The enzyme characteristic was done by determining the stability of enzyme to temperature and pH, and the enzyme kinetic parameter was also observed. The screening showed that cassava sugar syrup is the best alternative carbon source. The optimum concentration of cassava glucose syrup is at 10% of glucose levels, which will produce TvDAAO with the activity equal to 166.8861 U/g yeast cell dry weight. The enzyme characteristics stable at 4-10°C and pH 8, with Vmax value was 0.007 µmol/minute and KM was 78 mM. The used of cassava glucose syrup does not require any additional nitrogen source and it is became the advantageous of CGS as an alternative carbon source in terms of efficiency and economical of TvDAAO production.

Keywords— Cassava Glucose Syrup, D-Amino Acid Oxidase, Trigonopsis variabilis.

I. INTRODUCTION

There are two main groups of cephalosporin antibiotics; the first one is penicillin (G or V) derivative, the second is a cephalosporin C (CPC) derivative. The penicillin-derived products are mainly based on 7-Amino-desacetoxy cephalosporanic acid (7-ADCA) and the CPC-derived was 7-aminocephalosporanic acid (7-ACA). Active semi-synthetic cephalosporin are mostly derived from 7-ACA, whereby the 7-ACA issued as a precursor for the synthesis of the active pharmaceutical ingredient (API). CPC can be converted to 7-ACA in two ways, either chemically or an enzymatically [1]. CPC conversion in two enzymatic steps has become industrial standard for 7-ACA production. Two principle enzymatic routes are proposed (Fig.1), one-step hydrolysis of CPC with a CPC acylase (CA) and two-step cleavage with D-amino acid oxidase (DAAO) and glutaryl acylase (GAC) [2]. Hydrolysis of cephalosporin C into 7-ACA cannot be achieved as a one-step process since no enzyme with such type of activity has been discovered so far [3]. Recent study of CPC acylase was reported by Ma et al. [4].

The D-amino acid oxidase (DAAO, EC 1.4.3.3) is a flavoenzyme that can catalyzes the oxidative deamination of d-amino acids to produces α-keto and ammonia. DAAO plays a role in the production of α-keto acid, which is a useful therapeutic agent for the treatment of chronic uremia [5]. The main use of DAAO is in bioconversion of cephalosporin C (CPC) to 7-ACA. DAAO can be found in mammalian organs, mainly in the kidneys. DAAO can also be produced from the following types of microorganism, such as the yeasts Trigonopsis variabilis [6], Rhodotorula gracillis [7] and Candida tropicalis [8], the fungi Neurospora crassa [9]; Rhodosporidium spec. [10], Fusarium solani [11]. Only two enzymes, namely DAAO from Rhodotorula gracilis and Trigonopsis variabilis, have been developed into an industrial biocatalyst. Trigonopsis variabilis DAAO (TvDAAO) has the highest catalytic activity for CPC oxidation that's practical importance was reported[12].

Currently, Indonesia is still importing and spending a lot of money on various types of antibiotic raw materials for the use of local antibacterial industry and research purposes. This is due to the absence of intensive efforts made to produce raw materials of drugs using local resources. The production of antibiotic, such as penicillin and

cephalosporin industry, can be made by fermentation process. Agro industrial residuals have potentially been used as substrate in fermentation, not only for the production of enzymes but also others secondary metabolites. Cane molasses, an important residue of the sugar industry, have the potential as a cost-effective carbon source that could serve as nutrients for industrial enzyme-producing microorganisms, especially filamentous fungi [13]. Meanwhile, the use of other agro industrial residual, such as cassava glucose syrup and sorghum syrup, is limited or nonexistent. Countries like Indonesia with the abundant of the agro industrial residual have great potential to utilize it maximally.

This study aims to screen out the best alternative carbon sources that can be utilized in TvDAAO production, and to study the effect of the additional nitrogen sources, so that the TvDAAO production process becomes more efficient and economical. This study also to optimize the concentration of the best alternative carbon source.
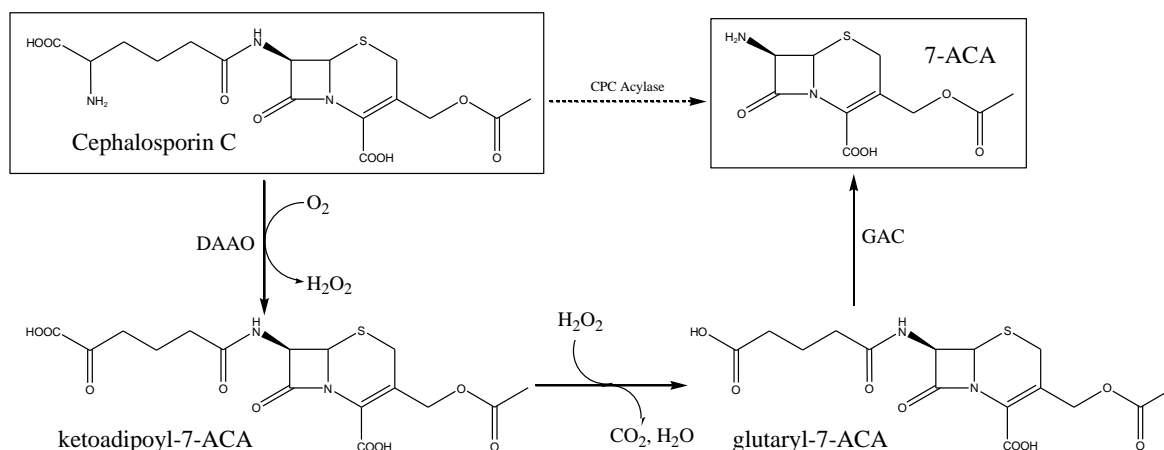

Fig. 1 : Enzymatic cleavage of CPC into 7-ACA by CPC acylase (upper, dashed line) and by two enzyme using D-Amino acid oxidase (DAAO) and Glutaryl Acylase (GAC) (lower, solid line).

II. MATERIALS AND METHODS

1. Materials

The microorganism used during the study was yeast, Trigonopsis variabilis was used as source of the enzyme, obtained from Biotechnology Collection Center-BPPT (Serpong, Indonesia). Molasses were obtained from Sragi Sugar Factory (Pekalongan, Central Java). Cassava glucose syrup were obtained from PT. Rejo Madusari (Pati, Central Java). Fructose sorghum syrup was obtained from PT. Sedana Agro (Sleman, Yogyakarta). DL-alanine were purchased from HiMedia. Glucose, K2HPO4, KH2PO4, MgSO4.7H2O, NaCl, CaCl2, MnCl2.4H2O, ZnSO4.7H2O, CuCl2.3 H2O, H3BO3, FeCl3.6 H2O were purchased from Merck (Darmstadt, Germany). The other chemicals, such as thiamin, biotin, o-phenylenediamine, horseradish peroxidase were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). All experiments were carried out in triplicate.

2. Culture medium and cultivation conditions

The strain was maintained in yeast malt medium. The cultures were kept at 4°C and subculture at regular intervals of 30 days.

Inoculum were prepared by transferring 1 mL suspension of the organism (OD600 = 1.0 – 1.2) from the slant culture to 50 mL pre culture medium (pH 6) in 250 mL Erlenmeyer flask containing glucose 22 g/L, DL-alanine 4 g/L, K2HPO4 2 g/L, KH2PO4 0.21 g/L, MgSO4.7H2O 0.5 g/L, NaCl 0.1 g/L, CaCl2 0.1 g/L, MnCl2.4H2O 0.105 g/L, ZnSO4.7H2O 0.0231 g/L, CuCl2.3 H2O 0.042 g/L, H3BO3 0.105 g/L, FeCl3.6 H2O 0.0714 g/L, 0.24 mg/L thiamin and 0.02 mg/L biotin. Incubation was carried out in orbital shaker at 30°C and 200 rev/min for 24 h for inoculum development.

Production of TvDAAO was carried out by transferring 8% inoculum (OD600 = 0.7 – 0.8) to 50 mL production media(pH 6) in 250 mL Erlenmeyer flask containing glucose 32 g/L, DL-alanine 6.2 g/L, K2HPO4 2 g/L, KH2PO4 0.21 g/L, MgSO4.7H2O 0.5 g/L, NaCl 0.1 g/L, CaCl2 0.1 g/L, MnCl2.4H2O 0.105 g/L, ZnSO4.7H2O0.0231 g/L, CuCl2.3 H2O 0.042 g/L, H3BO3 0.105 g/L, FeCl3.6 H2O 0.0714 g/L, 0.24 mg/L thiamin and 0.02 mg/L biotin.

Cassava glucose syrup and DL-alanine was sterilized using 0.2 µm filter. The other components were sterilized in the autoclave at 121 °C for 15 min. Incubation was carried out in an orbital shaker at 30°C and 200 rev/min for 24 h for inoculum development.

3. Fermentation and permeabilization of cell mass

Initially, the production of TvDAAO was carried out in fermentation at 30°C and 140 rev/min for 72 h. The cells were harvested and neutralized to pH 6.0 using potassium hydroxide, then centrifuged at 10,000 g at 4°C for 10 min to obtain cell mass, and then washing with potassium phosphate buffer (pH 8.0). About 1 mL of the cell suspension were analyzed gravimetrically to obtain the cell mass.

The rest of washed cells were suspended using the same buffer. The suspension cells were permeabilized using 5% toluene-ethanol (1:1) and held for 1 h at 37°C. The permeabilized cells were used for enzyme assay.

4. Enzyme Assays

The DAAO activity were measured by o-phenylenediamine/horseradish peroxidase coupling assay[14]. The reaction mixture contained 30 mM D-alanine, 0.03% o-phenylenediamine, 1700 U horseradish peroxidase and DAAO of interest in 100 mM potassium phosphate buffer (pH 8.0). The reaction was monitored by an increase in absorbance at 450 nm for 3 min at 25°C.

One unit of enzyme activity was defined as the enzyme needed to produce 1 micromole of H_2O_2 per min at 25°C and pH 8.0.

5. Screening an alternative carbon source and the effect of the additional nitrogen source

The screening of alternative carbon sources were done by replacing glucose with molasses, CGS and SFS, without or with the addition of a ammonium sulphate (0.5%) as a nitrogen source. The sugar levels of alternative carbon source that being used is equal to glucose concentration (3.2%). Sugar levels in alternative carbon sources were analyzed using dinitro salicylic acid (DNS) method. Measured sugar levels are equivalent to glucose.

6. Optimization of the best alternative carbon source concentration

The best alternative carbon source was selected for optimization. The optimization was carried out at various concentrations.

7. Enzyme characterization

The enzyme characteristic was done by determining the stability of enzyme to temperature and pH, and the enzyme kinetic parameter was observed.

The temperature stability of TvDAAO was studied in a 0.1-M potassium phosphate buffer, pH 8.0. A series of 0.5 ml plastic test tubes containing 100 μl of the enzyme solution were prepared for each experiment. The tubes were placed to a preheated to the desired temperature water thermostat (temperature control accuracy ± 0.1°C). The test tubes were sampled one by one after fixed time

intervals, rapidly cooled for 1-2 min in ice, and the enzyme activity was measured as described above.

The pH stability of TvDAAO was studied in a 0.1-M potassium phosphate buffer, pH 8.0. A series of 0.5 ml plastic test tubes containing 100 μl of the enzyme solution were prepared for each experiment. The pH was adjusted with H3PO4/KOH to the desired pH. The tubes were placed to a preheated at 25°C water thermostat. The test tubes were sampled one by one after fixed time intervals, rapidly cooled for 1-2 min in ice, and the enzyme activity was measured as described above.

The enzyme kinetic parameters were done by determining the maximum reaction rate (Vmax) and Michaelis constant (KM), the concentration of the corresponding D-amino acid was varied from 10 to 300mM. The concentration of the active enzyme was measured as described above. The enzyme kinetics parameters (KM and Vmax) were determined by plotting the relationship graph between 1/V and 1/S, then determined the values of KM and Vmax based on the Lineweaver-Burk curve equation.

III. RESULTS AND DISCUSSION

1. Screening an alternative carbon source and the effect of the additional nitrogen source

The screening of an alternative carbon sources were done with a one-at-a-time strategy. Types of alternative carbon sources used are molasses, cassava glucose syrup and sorghum fructose syrup. The screening was done without or with the additional of a nitrogen source. The additional nitrogen source selected was ammonium sulfate, in which ammonium sulfate was one of the best source of nitrogen for yeast culture [15; 16]. The results of the screening were shown in Table 1.

Table 1 shows the TvDAAO activity resulting from fermentation using various types of carbon sources as nutrients in the production process. From various carbon source, glucose with ammonium sulfate was found to be the most suitable to induce the TvDAAO production. Glucose requires no extra step to enter in glycolytic pathway. However, for better results, the uses of glucose need an additional nitrogen source, where nitrogen plays a role in the formation of amino acids for the production of enzymes. It is made to be less efficient and uneconomical. It is different when using agro-industry residues as an alternative carbon source. The best alternative carbon source is cassava glucose syrup, with enzyme activity obtained is 73.5079 U/g. Cassava glucose syrup contains glucose as the main sugar [17], which is glucose was the best carbon source for TvDAAO production. The use of cassava glucose syrup as a carbon source can be done without the addition of additional nitrogen sources. This is because the amount of nitrogen required during the fermentation process has been fulfilled from the cassava

sugar syrup itself and from DL-Alanine. Portilho [18] mentioned that the C/N ratio in cassava glucose syrup was above 20. The use of an additional nitrogen source will decrease the resulting TvDAAO, because nitrogen can lead to the formation of ammonium, where excessive ammonium can cause cell death[19]. This is an advantage in the use of cassava glucose syrup as an alternative carbon source of glucose substitutes, because cassava glucose syrup can act as a source of carbon as well as a source of nitrogen, so the production becomes more efficient and economical.

Table 1. Screening of various types of carbon source, with or without an additional NH4(SO4)2

| Carbon Source | Enzyme activity (U/g yeast cell dry weight) | |
	without NH$_4$(SO$_4$)$_2$	with NH$_4$(SO$_4$)$_2$
Glucose	52.5286 ± 0.3880	100.9528 ± 0.7311
Molasses	47.9158 ± 0.7447	0.3421 ± 0.0126
Cassava glucose syrup	73.5079 ± 1.4246	0.2666 ± 0.0080
Sorghum fructose syrup	42.9478 ± 0.6666	0.5628 ± 0.0336

2. Optimization of cassava glucose syrup concentration

The productivity of cassava glucose syrup can still be improved by increasing the concentration of cassava glucose syrup (Figure 2). The concentration of cassava glucose syrup is measured as the glucose content contained in the cassava glucose syrup. Figure 2 shows that the highest enzyme activity (166.8861 U/g yeast cell dry weight) was obtained at 10% of glucose levels in cassava glucose syrup. The productivity of the use of cassava glucose syrup in TvDAAO production can still be optimized using a response surface method (RSM), which will be discussed in another article.

3. Enzyme characterization

The result of stability test of temperature and pH can be seen in Figure 3 and 4. The enzyme was stable at 4-10°C (Fig. 3). The results obtained are similar to those reported by Kubicek-Pranz & Rohr [20], which state that the TvDAAO enzyme is stable at 4°C. Meanwhile, on the stability test of pH (Fig. 4), it is known that the enzyme is quite stable at pH 8. At pH 5, the TvDAAO enzyme will precipitate because the isolation point of TvDAAO enzyme is at pH 5.1[21].

The enzyme kinetics parameters, including Vmax and KM, are determined by the Lineweaver-Burk curve equation (Fig. 5). The results obtained Vmax value of 0.007 µmol / minute and KM of 78 mM. The value of KM obtained is not significantly different from the value of KM obtained by Szwajcer & Mosbach [22] that is equal to 76 mM.

IV. CONCLUSION

Cassava sugar syrup was the best alternative carbon source to be utilized in TvDAAO production by fermentation process. Cassava sugar syrup can act as a source of carbon as well as a source of nitrogen, so that the production of TvDAAO become more efficient and economical. The optimum concentration of cassava glucose syrup is at 10% of glucose levels, which produced TvDAAO having activity equal to 166.8861 U/g yeast cell dry weight. The enzyme stable at 4-10°C and pH 8, with Vmax value was 0.007 µmol/minute and KM was 78 mM.
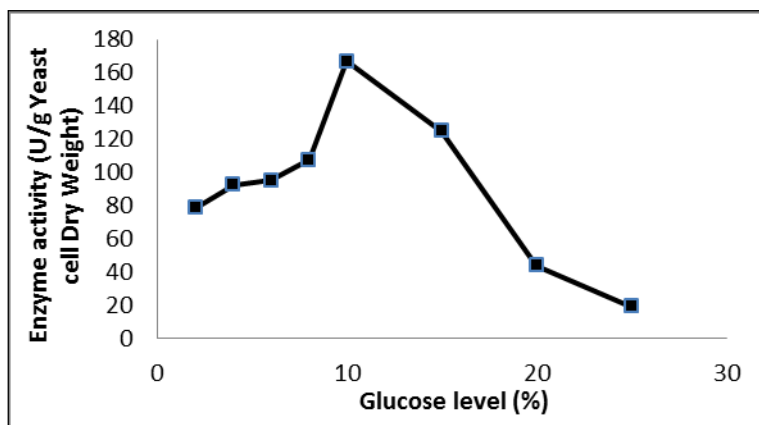

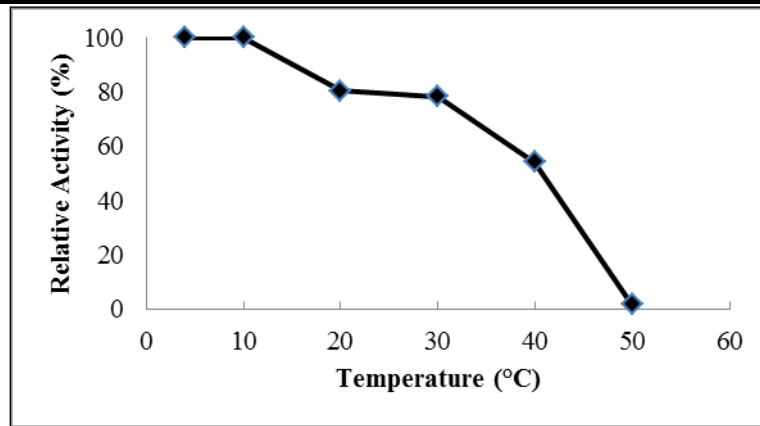*Fig. 2 : Effect of various concentration of cassava glucose syrup without additional nitrogen source*
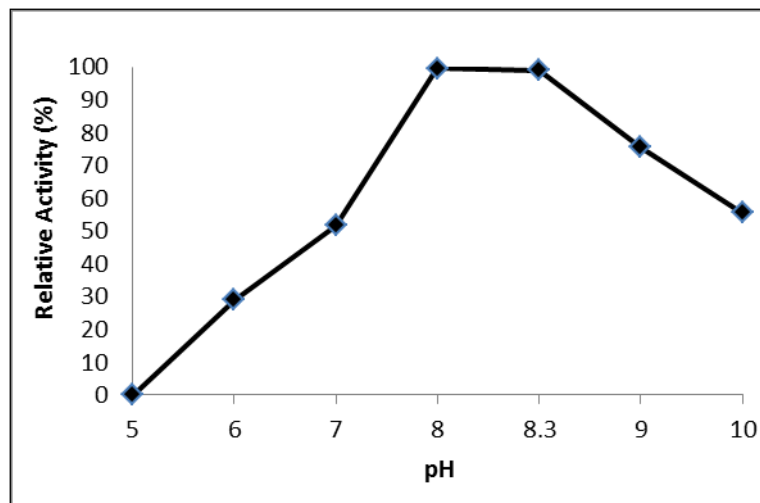
Fig. 3 : Stability of the enzyme to temperature

Fig. 4 : Stability of the enzyme to pH

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