ENHANCED PRODUCTION OF EXTRACELLULAR URATE OXIDASE BY *ASPERGILLUS FLAVUS*

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ABSTRACT

Urate oxidase or uricase is an enzyme that catalyses the oxidation of uric acid to allantoin and plays important rule in purine metabolism. The first important application discovered for uricase was in clinical biochemistry as diagnosis reagent for measurement of uric acid in blood. The precipitation of uric acid can leading to gout symptom. The main purpose of this research is to optimize the culture condition for maximum uricase production by *Aspergillus flavus*. The parameters studied were pH (range of 4 to 8), the sucrose concentration (10g/l to 50 g/l) and the agitation rate (100 to 300 rpm). The *Aspergillus flavus* were inoculated in yeast extract with sucrose and incubated for 24 hours at 200 rpm and 30°C. The maximum enzyme activity obtained from the experiment is 0.03974 U/ml at pH 6, 200 rpm and 30 g/l sucrose concentration. Since the optimum conditions were obtained, the production of uricase can be enhanced and world demand on uricase can be fulfill in the future.

MENINGKATKAN PENGELUARAN EKSTRASELULAR URATE OXIDASE OLEH *ASPERGILLUS FLAVUS*

ABSTRAK

Urate oksidas atau uricase merupakan enzim yang menjadi pemangkin pengoksidaan asid urik ke alantoin dan memainkan peranan penting dalam metabolisme purin. Aplikasi pertama yang penting ditemui untuk uricase adalah dalam biokimia klinikal sebagai reagen diagnosis untuk mengukur asid urik dalam darah. Pemendakan asid urik boleh membawa kepada gejala penyakit gout. Tujuan utama kajian ini adalah untuk mengoptimumkan keadaan kultur pengeluaran uricase maksimum oleh *Aspergillus flavus*. Parameter yang dikaji ialah pH (julat 4-8), kepekatan sukrosa (10g / l hingga 50 g / l) dan kadar pengadukan (100-300 rpm). *Aspergillus flavus* telah diinokulasi dalam ekstrak yis dengan sukrosa dan dieram selama 24 jam pada 200 rpm dan 30 °C. Aktiviti enzim maksimum diperolehi dari eksperimen tersebut adalah 0, 03974 U / mL pada pH 6, 200 rpm dan kepekatan sukrosa 30g/l. Oleh kerana keadaan optimum telah diperolehi, pengeluaran uricase boleh dipertingkatkan dan permintaan dunia pada uricase boleh dipenuhi pada masa depan.

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LIST OF SYMBOLS

μ	Specific growth rate
μ_m	Maximum specific growth rate
K_s	Saturation constant

LIST OF ABBREVIATIONS

ALL	Acute Lymphoblastic Leukemia
DNS	Dinitrosacyclic method
MARDI	Malaysian Agricultural Research and Development Institute
PDA	Potato dextrose agar
S	Substrate concentration
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

CHAPTER 1

1.0 INTRODUCTION

1.1 Background of Proposed Study

Urate oxidase or uricase is an enzyme that catalyses the oxidation of uric acid to allantoin and plays important rule in purine metabolism. This most find in vertebrates. The first important application discovered for uricase was in clinical biochemistry as diagnosis reagent for measurement of uric acid in blood. The precipitation of uric acid can leading to gout symptom. Gout treatment generally includes allopurinol, however with direct injection of urate oxidase allow much more rapid resorption of urate nephrolithiases. The minimum plasma concentration of uric acid was significantly lower after treatment with uricase compared to allopurinol treatment. In this case, uricase is the only substrate that can bind to uric acid (Atalla *et al.*, 2009).

The urate oxidase can be produce by many type of microorganism. About nineteen of strains can produce urate oxidase. Atalla *et al.* (2009) stated that urate

oxidase can be produced from *Gliomastix gueg.* Chen *et al.* (2008) proved that urate oxidase can be produce from recombinant *Hensula polymorpha* with three copies of expression cassette and selected for process optimization for the production of recombinant enzyme. Beside that, bacteria like *Microbacterium* also can produce urate oxidase through purification (Kai *et al.*, 2007). However, urate oxidase is commercially produced by *Aspergillus flavus* (Atalla *et al.*, 2009).

1.2 Problem Statement

The uricase is the better way use to treat gout as it allows much more rapid resorption of urate nephrolithiases compared to previous method which used allopurinol. The uricase is better because it gives more rapid resorption to the gout's patient. The researchers also found that, in their study of more than 5,000 people, about 21.4 percent had high levels of uric acid in their blood, which is known to be a risk factor for developing gout (Retrieved on 27th April 2012 from http://www.scientificamerican.com/article.cfm?id=gout-on-the-rise). The number of gout's patient increasing rapidly, however the production uricase nowadays still not fulfil the demand.

Even though uricase had been produced by fermentation in previous study, the optimum condition that can enhance the maximum production of urate oxidase still in study. The best agitation rate, optimum pH value and carbon source concentration is still unknown.

The urate oxidase can be produced from many types of fungal, yeast and bacteria. For example, bacteria like *Bacillus subtilis* can produce uricase. However, this bacterium is not thermal stable and it will lost their activities in short period at temperature higher than 60 °C (Zhou *et al.*, 2005). Fungal strain is the best microorganism because it is easy to manage and less sensitive. Beside that, the fungal strain is more stable than other microorganism that can produce uricase. The productions of uricase from yeast and bacteria source are highly cost and less production. *Aspergillus flavus* is one of the fungal strain that easily available and less expensive compared to other fungus strains.

1.3 Research Objective

1.3.1 To screen the optimum parameters (pH, agitation rate and sucrose concentration) in order to enhance the production of uricase

1.4 Scope of Proposed Study

The research is focusing on fermentation condition that influences the production of uricase by *Aspergillus flavus* in shake flask cultures. In order to find the optimum condition, the experiment was carried out using one factor at a time technique. The concentration of sucrose (ranged from 10 to 50 g/l), pH of the fermentation medium

(ranged from 4 to 8) and agitation rate (ranged from 100 to 300 rpm) were studied one by one in a selected range determined form literature review.

1.5 Significance of Proposed Study

The importance of the proposed research is to enhance the maximum production of uricase and to fulfil the market demand. Theoretically, the uricase give the better effect on gout's patient compared to medicine used nowadays like allopurinol because direct injection of urate oxidase allow much more rapid resorption of urate nephrolithiases. Beside that, it helps the better life of humankind in the future. The available medicine nowadays is too expensive and not all people can afford the price of that medicine. Therefore, the fungal strain use in the research is the less expensive and the method use is the research also less expensive in order to produce medicine that all level of people can afford.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Uricase

Uricase can be produce from many types of fungus such as *Gliomastix gueg, Microbaterium* and *Hansenula polymorpha (* Atalla *et al.,*2009, Kai *et al.,*2008, Chen *et al.,*2008*)*. The first application discovered for uricase was in clinical biochemsistry as diagnostic reagent for measurement of uric acid in blood and other biological fluids (Atalla *et al.*, 2009). Uricase, which catalyse the oxidation breakdown of uric acid, belong to a group of enzyme in the purine degradation pathway in animals, plants, fungi, yeast and bacteria (Kai *et al.,* 2008). In December 1991, pharmacodynamic study on methotrexate and mercatopurine before conventional remission induction therapy with newly diagnosed acute lymphoblastic leukemia (ALL) with allopurinol used to treat hyporuricemia. After finding the effect of allopurinol, the uricase is replaced to treat cancer patient (Pui *et al.*, 1997).The urate oxidase can acts as a catalyst in the enzymatic

oxidation of uric acid to allantion, a readily excreted metabolite that five- to 10- fold more soluble than uric acid (Pui *et al.*, 2001).

2.2 Microbial Production of Uricase

Chevalet *et al.* (1992) stated *Aspergillus flavus* can be the resistance to drugs, allow for transformation of any fungal strain that is sensitive to such inhibitors. Beside that, *Aspergillus flavus* is concerned with the complementation of genetic defects, among which auxotrophy for arginine or uridine and the mutation affecting nitrate assimilation are well known.

Atalla *et al.* (2009) stated that microbial enzyme is inducible and therefore, the presence of uric acid or some other inducer in the medium is necessary for enzyme production. Several microbial sources of uricase have been proposed for this clinical indication, only one has actually been used commercially under the trade mark of uricozyme and is isolated and purified from *Aspergillus flavus*.

Beside that, Lotfy (2008) studied that *Bacillus thermocatenulatus* strain can produce uricase. The aim of the study is to investigate the ability of *Bacillus thermocatenulatus* as a novel uricase producer and to optimize the enzyme productivity. The strain is quite stable because the aeration rate does not give any changes on production rate and growth of the strain.

Yazdi *et al.* (2006) stated there is no report exists on the production uricase by *Mucor hiemalis*. However, it is necessary to screen the new sources for production of uricase which more economical, and which may have unique properties to expand its usefulness. Therefore, he proved that *Mucor hiemalis* can produce uricase. The *Mucor hiemalis* is one of common soil fungi. The study describes the isolation of a strain capable of producing relatively high levels of uricase in short time. Optimization of medium composition and relevant conditions for enzyme activity are also clarified.

2.3 Monod equation

The Monod equation describes substrate-limited growth only when growth is slow and population density is low. Figure 2.1 is the relationship of specific growth rate to substrate concentration often assumes the form of saturation kinetics. Assume that a single chemical species, S, is growth-rate limiting which mean increase in S influences growth rate, while changes in other nutrient concentrations have no effect (Shuler and Kargi, 2002). This is quite similar to the Michaelis-Menten kinetics for enzyme reactions. Since it applied to cellular systems, this kinetics can be described by the Monod equation:

$$\mu = \frac{\mu_m S}{K_s + S} \qquad (2.1)$$

Where; μ = specific growth rate, μ_m = maximum specific growth rate,S= substrate concentration (g/l) and K_S = saturation constant.

Maximum specific growth rate, μ_m is when S$\gg K_S$. If endogeneous metabolism is unimportant, then $\mu_{net} = \mu$. the constantK_S is known as half-velocity constant and is equal to the concentration of the rate limiting substrate when specific rate of growth is equal to one-half of the maximum. That is, $K_S = S$ when $\mu = 1/2\mu_m$. In general, $\mu = \mu_m$ for S$\gg K_S$ and $\mu = (\mu_m/K_S)S$ for S$\ll K_S$. The Monod equation is semiempirical; it derives from the premise that a single enzyme system with Michaelis-Menten kinetics is responsible for uptake of S, and the amount of that enzyme or its catalytic activity is sufficiently low to be growth-rate limiting.

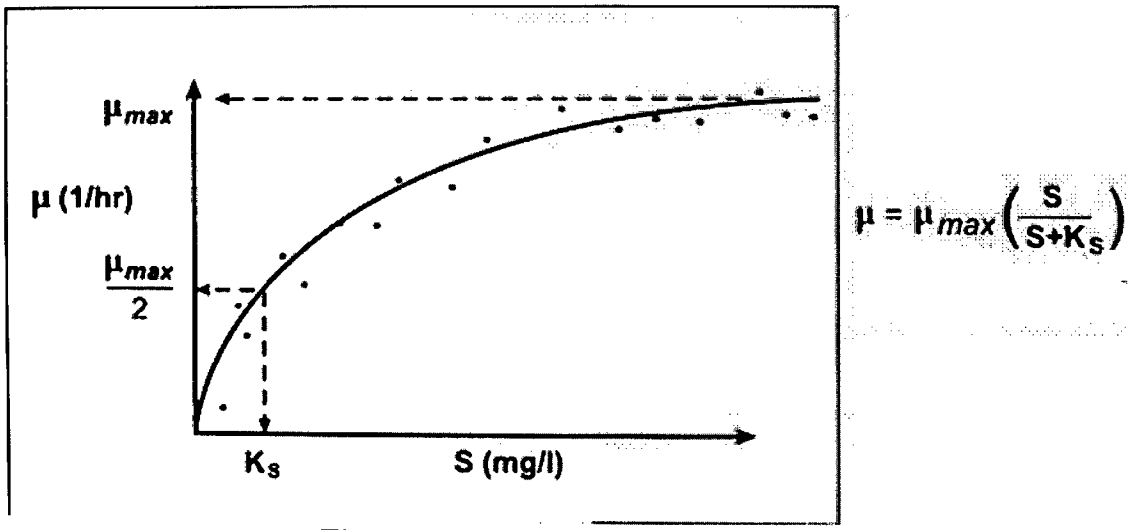

Figure 2.1 Substrate-limited growths.
(Source: Shuler and Kargi, 2002)

2.4 Factors that Enhance the Production of Uricase

For the effect of pH of uricase production by *Aspergillus flavus*, Tohamy and Shindia (2002) and Yazdi *et.al* (2006) agree that pH 6 was the optimum pH for *Aspergillus flavus*. Beside that, Ammar (1988) reported at pH 9.2 was the optimum pH for the best production. Chen *et al.* (2008) stated that the best specific growth rate was obtained at pH 5.5 and highest cell-specific uricase production was achieved at pH 6.5, a pH control strategy of switching pH value from 5.5 in cell growth phase to 6.5 in induction phase was used to improve uricase production. Anderson and Vijayakumar (2011) reported highest uricase activity was attained at pH 8.5 and 35 ^{o}C using *Pseudomonas aeruginosa*. Beside that, Yazdi *et al.* (2006) reported that maximum production was achieved at pH 6 by *Aspergillus terreus*.

The effect of carbon source concentration on uricase production by *Aspergillus flavus,* Atalla *et al.* (2009) agreed with Abd El Fattah and Abo Hamed (2002) that *Aspergillus flavus* produced the highest amount of uricase in the medium containing sucrose. Therefore, the best carbon source is sucrose. However, the best concentration of sucrose is still in study.

Based on literature review, it was found out that until today, there is no study on the effect of agitation rate for uricase production. Therefore, this study will focus on the effect of agitation rate (rpm) at range 100 to 300.

2.5 Production of Uricase

New crystal-packing contacts in relation to the content of active site can produce uricase from *Aspergillus flavus*. Expression of the recombinant enzyme *Saccharomyces cerevisiae* followed by a new purification procedure allowed the crystallization of both unliganded and liganded enzyme utilizing the same condition but in various crystal forms. The inhibitor used is chemical (Pascal *et al.*, 2005).

The uricase also can be produced by extraction. Anderson and Vijayakumar (2011) stated that uricase can be produced by extraction using *Pseudomonas aeruginosa*. Then, uricase was purified by ammonium sulphate precipitation. The purified enzyme was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

The uricase produced from fermentation method by using *Gliomastix gueg*. The fermentation medium used in inoculated flask are uric acid, Czapek dox and yeast extract sucrose. The inoculated flasks were incubated on a rotary incubator shaker at 150 rpm for 8 days at 30 °C after which the mycelium of each isolate was collected by centrifugation at 5000 to 6000 rpm for 15 min at 4°C (Atalla *et al.*,2009).

2.6 Application of Uricase

The most commercial of uricase application is as an enzyme participating in the purine breakdown pathway, catalyzing the oxidation of uric acid in the presence of oxygen to allantoin and hydrogen peroxide (Anderson and Vijayakumar, 2011). It has beneficial uses both in vitro and in vivo. Beside that, uricase is useful for enzymatic determination of urate in clinical analysis by coupling with 4-aminoantipyrine-peroxidase system (Lotfy, 2008). Determining the urate concentration in blood and urine is required for the diagnosis of gout as urate accumulation is a causative factor of gout in humans.

The first important application discovered for uricase was in clinical biochemistry as diagnostic reagent for measurement of uric acid in blood and other biological fluids (Atalla *et al.*, 2009). Uricase acts as a catalyst in the enzymatic oxidation of uric acid to allantoin, a readily excreted metabolite that is five- to 10-fold more soluble than uric acid. It is an endogenous enzyme in most mammals, but not in humans (Pui *et al.*, 2001).

Anderson and Vijayakumar (2011) investigated the purification and optimization of uricase for protein structure analysis and to carry out animal studies in the future for drug development. Lotfy (2008) stated that uricase also can be used as protein drug for treatment of hyperuricemia as Rasburicase.

CHAPTER 3

3.0 METHODOLOGY