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The Effect of Inducers, Nitrogen Compound and Glucose on the Uricase Production by *Lactobacillus plantarum* Dad-13

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Abstract:
Uricase plays an important role in the reducing of serum uric acid in human and can be used as a diagnostic reagent. This research aimed to evaluate the effect of inducers, nitrogen and glucose in the medium on intracellular uricase production by Lactobacillus plantarum Dad-13. Pepton Glucose Yeast Extract (PGY) with or without glucose and pepton was used as a medium for uricase production while adenine, guanine and uric acid were added into the medium as an inducer. The result showed that uricase produced by L. plantarum Dad-13 is an inducible enzyme which needs uric acid as an inducer while adenine and guanine can not induce uricase production. Glucose and peptone play an important role on uricase production by L. plantarum Dad-13 while peptone is needed for uric acid transportation into the cell. The highest of uricase production resulted from PGY medium and the lowest resulted from PGY medium without peptone supplementation due to lower intracellular of uric acid.

Keywords: *uricase, intracellular of uric acid, nitrogen, glucose*

1. Introduction

Uricase is an oxidoreductase enzyme which catalyzes the oxidation of uric acid into allantoin, and hydrogen peroxide. Allantoin has a high solubility than uric acid so that more easily excreted from the body (Sherman *et al*. 2008); (Khade and Srivastava, 2015). Uricase conserved in many species ranging from microorganism to mammals but absent in human. Uricase is a promising enzyme that used for determination of uric acid levels in clinically and as a protein drug for the treatment of hyperuricemia which has high specificity toward uric acid (Coiffier *et al*., 2003; Azab *et al*., 2005).

Uricase used in the clinical purpose is produced only from *Aspergillus flavus*, under the trademark of uricozyme. Recently, the investigation has been focused on the production of uricase from several microbial sources. *P. aeruginosa*, *B. thermocatenulatus, B. subtilis* and *Lactobacillus plantarum* produced extracellular uricase (Saeed *et al*., 2004; Lotfy, 2008; Amirthanathan and Subramaniyan, 2012); while *Proteus vulgaris, Streptomyces graminofaciens, S. albidoflavus* (Azab *et al*., 2005)*, Microbacterium* (Zhou *et al*. 2005) and *L. plantarum* (Iswantini *et al*., 2009) produced intracellular uricase. *L. plantarum* is lactic acid bacteria that has the ability as probiotic source. On the other hands, from our previous research there were several candidates of probiotic strains (Purwandhani and Rahayu, 2003; Rahayu *et al*., 2011; Rahayu *et al*., 2015).

L. plantarum Dad-13 is lactic acid bacteria that has the ability as probiotic source which tolerant into gastrointestinal system (Ngatirah *et al*., 2000; Rahayu *et al*., 2011; Rahayu *et al*., 2015) and produced intracellular uricase (our previous study). The development of probiotics that produce uricase and administrate as oral food with high stability in gastrointestinal system is a promising potential therapy for the prevention of hyperuricemia. However, orally-administration uricase for treatment hyperuricemia remains challenging. Low pH, pepsin, pancreatic and bile salt in gastrointestinal track induced protein unfolding and propensity cleaved peptide bond resulting in enzyme inactivation (Fuhrmann and Leroux, 2014). Probiotics have an ability produce several exogenous enzymes

(amylase, lipase, protease, cellulase) which would assist endogenous enzymes of host in hydrolysing nutrients (Putra and Widanarni, 2015). β-galactosidase and sacrosidase remained have activity in the gastrointestinal system (Fuhrmann and Leroux, 2014; Martini *et al*., 1987). The cell membrane and cell wall could maintain the pH of intracellular cytosolic that protected the uricase from the hydrolytic activity of the gastric protease.

In various microorganisms, uricase synthesis is regulated by the compounds of medium (Abdel-Fatah *et al*., Abdullah *et al.*, 2015; Azab *et al*., 2005). (Bongaerts *et al*. 1978)). Watanabe *et al*., (1976) reported that either limitation of nitrogen and carbon sources released inhibition of uricase formation in resting cells. Purine and purine derivate can induce uricase by some microorganism and uric acid acted as the best inducer (Azab *et al*. 2005). Furthermore, glucose has a negative effect on uricase activity (Nahm and Marzluf 1987; ˈNanda et al., 2012). Since uricase production was controlled by a metabolite repression of nitrogen and carbon source in the present of purine derivate, the production of uricase by *L. plantarum* Dad-13 in various composition mediums which were repressed in glucose and nitrogen source and purine derivate (adenine, guanine and uric acid) was needed evaluation.

This study aims to investigation the effect of inducers, nitrogen and glucose in the medium on uricase production by *Lactobacillus plantarum* Dad-13 in order to find the higher of uricase production.

2. Materials and Methods

2.1. The Strain

L. plantarum Dad-13 which was isolated from fermented milk, and obtained from Food and Nutrition Culture Collection (FNCC), Center for Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta, Indonesia was used for uricase production in this study. Strain was stored in a medium containing 10% glycerol and 10% skim milk with the ratio of 1:1 (v/v) in the 1.5 mL polyethylene sterile cap tube and stored at -40°C as a stock.

2.2. Preparation of Culture Starter

The strain was cultivated by adding 0.1 mL of stock culture in 10 mL of PGY medium at 37°C for 18 h. The cultivation of the culture was done twice.

2.3. The Role of Uric Acid, Adenine and Guanine towards Production uricase

In order to determine the role of uric acid, adenine, and guanine against uricase production by *L. plantarum* Dad-13, PGY medium was used in this study. PGY medium composed of glucose (Merck) 1%, yeast extract (Merck) 1%; peptone (Merck) 0.5%; beef extract (Merck) 0.2%, sodium acetate (Merck) 0.014%; Tween-80 (Merck) 1%; and 0.5% salt solution. Salt solution composed of magnesium sulfate (Sigma) 0.2%; manganese sulfate (Sigma) 0.2%; ferry sulfate (Sigma) and 0.2% sodium chloride (Merck) 0.2%. 0.5% starter culture and 0.15% of uric acid, adenine or guanine was added as inducers into 30 ml of medium PGY. Incubation was performed at 37° C for 22 hours. At the end of incubation cells were harvested and followed by preparation of uricase.

2.4. The effect of Nitrogen Compound and Glucose on uricase Production by L. plantarum Dad-13

L. plantarum Dad-13 was grown in 45 mL of PGY medium as a control that consisting of 1% glucose, 1% yeast extract, 0.5% peptone, 0.2% beef extract, 0.14% natrium acetate, 1% tween-80, 0.5% salt solution (Iswantini *et al*., 2009). PGY medium with glucose as well as peptone ommittion used as nitrogen and carbon repression medium. 0.15% of uric acid was added into the medium as an inducer while medium without uric acid was used as control negative for indusible enzyme. Incubation was done at 37ºC for 22 h for uricase production. After fermentation, cells were harvested and uricase activity was evaluated.

2.5. Preparation of Intracellular Uricase

After fermentation, a 30 mL of culture was centrifuged at 3000 rpm (4ºC) for 20 min to separate the supernatant. Bacterial cells were washed with 5 mL of 0.1 M sodium phosphate buffer, pH 7.0. The washing of bacterial cells were repeated twice (Kai *et al*., 2007). Disruption of cells were done by adding the quartz sand (150-212 µm) to the cell suspension and stirred vigorously for 10 min, with occasional cooling in the ice bath *(Carevic et al*., 2015). To separate the cell debris, the sample was centrifuged at 6000 rpm (4^{o}C) for 20 min and the supernatant used as crude intracellular. Uricase activity was evaluated as criteria.

2.6. Cell, uric acid in the cell and pH Enumeration

Cell, intracellular of uric acid, and pH of medium production of uricase were also analyzed after fermentation. The growth of cells was determined by pour plate count method. Intracellular of uric acid was determined by spectrophotometer at 293 nm (Chen and Gomes 1992). pH was determined by digital pH meter (Thermo scientific, Orion).

2.7. Uricase Assay

Uricase activity was determined according to Bergmeyer (1974) min at 37ºC. About 0.08 mL of crude uricase was added into a mixture of 3.09 mL of borate buffer pH 8.0 and 0.01 mL 3.57 mM uric acid. Mixture was incubated for 10 min at 37^{o}C. The reaction was stopped by boiling the mixture at 5 min. Besides, a mixture was boiled directly after addition of crude uricase as a reference. The absorbance was measured at 293 nm using spectrophotometer. The difference between the absorbance of the sample and the reference was equivalent to the decrease in uric acid during the enzyme reaction. One unit of uricase activity was equal to the amount of enzyme, which converts 1µmol of uric acid to allantoin per min at 37ºC.

3. Results and Discussion

3.1. The Effect of Inducers (Adenine, Guanine and Uric Acid)

The result of this research as shown in Tabel 1 showed that adenine, guanine and uric acid had no effect on the growth of *L. plantarum* Dad-13, but they had significantly effect on the uricase production, intracellular of uric acid, cells and pH of the medium.

Inducers	Uricase activity (U/mL culture)	Intracellular of uric acid (umol/mL culture)	Cells (log CFU/mL culture)	pH
Adenine	0.00 ± 0.00^{b}	1.76 ± 0.06^{c}	9.03 ± 0.02^{a}	3.72 ± 0.01^{b}
Guanine	0.00 ± 0.00^{b}	8.37 ± 0.10^{b}	9.13 ± 0.08^{a}	3.80 ± 0.01^{a}
Uric acid	1.07 ± 0.03^{a}	205.90 ± 1.90^{a}	9.59 ± 0.02^{a}	3.63 ± 0.01^{c}

Tabel 1: The effect of inducers on uricase activity, intracellular of uric acid, cells, and pH after fermentation in PGY medium supplemented with 0.15 % of inducer by L. plantarum Dad-13. It was incubated at 37°C for 22 h

Values are mean ± standard deviation and with different superscript letters in each row are significantly different ($p<0.05$). Values are mean ± standard deviation and with different superscript letters in each row are significantly different ($p<0.05$).

Uricase was only produced in the medium containing of uric acid as an inducer while adenine and guanine could not induce uricase production by *L. plantarum*-13. It was suggested that *L. plantarum* Dad-13 was unable to produce adenine and guanine deaminase which hydrolyzed adenine and guanine into uric acid as shown in the low concentration of intracellular uric acid. Machida and Nakanishi (2016) reported that since enzyme concerned in adenine catabolism, adenine deaminase may be a very week or just did not exist, thus *Enterobacter cloacae* had very low uricase activity. Azab *et al*., (2005) reported that uric acid is the best inducer for uricase production by *Streptomyces graminofaciens* while guanine is the worst

Harmayani *et al*. (2001) reported that *L. plantarum* Dad-13 reached stationary phase after 16 h of incubation in MRS medium, while in the PGY medium stationary phase was reached at 12 h of incubation. Uricase activity started at stationary phase on the growth of *L. plantarum* Dad-13 (not shown). In this phase, due to lack of nutrient, *L. plantarum* Dad-13 used uric acid as the source of carbon, nitrogen, and energy. Since uricase activity starting produced at the stationary phase, while adenine and guanine were not used by *L. plantarum* Dad-13, thus additional of uric acid, adenine and guanine had no effect on the cell amount.

The change of pH of PGY medium, which was supplemented by adenine, guanine and uric acid during incubation as shown in Tabel 1 showed that uric acid gave the lowest pH due to uric acid categorized as acidic compound while adenine or guanine was identified as purine base compound. The uric acid which was the best inducer for uricase production by *L. plantarum* Dad-13, furthermore used in the following study.

3.2. The Effect of Nitrogen Compound and Glucose Repression

Nitrogen compound and glucose in the medium had significant effect on uricase activity, cells, intracellular of uric acid and pH when uric acid supplemented into the medium, while un signifficant effect was observed in no supplementation of uric acid.

The addition of 0.15% uric acid into PGY medium with glucose or pepton omitled had significant effect on the enhancement of the cell while the addition of uric acid into PGY medium as a control medium had un significant effect (Figure1). It suggested that *L. plantarum* Dad-13 used uric acid as carbon, nitrogen and energy sources for growing of the cells when the cell growth in the minimum medium, meanwhile in the PGY medium, uric acid was used with uricase production by *L. plantarum* Dad-13 in the stationary phase (not shown), so the additional of uric acid into PGY medium had no significant effect on the cells. This research was accordance with Aly *et al*., (2013) whose reported that medium composition affected the growth of *Streptomyces exfoliatus* while there is no significant relantionship between bacterial growth and uricase production.

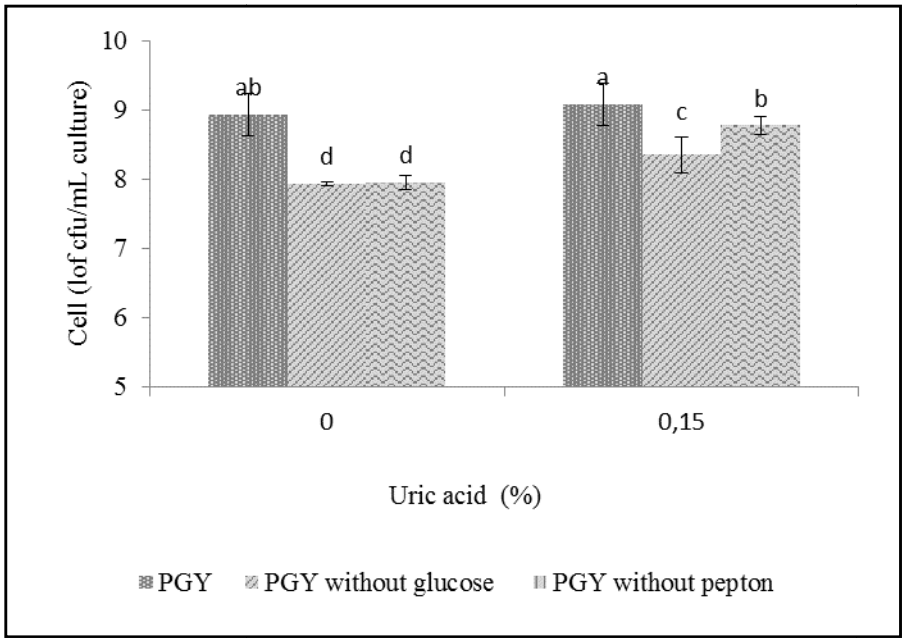

Figure 1: The effect of nitrogen compound and glucose in the PGY medium on cells after fermentation by L. plantarum Dad-13 at 37°C for 22 h.

The minimum growth of *L. plantarum* Dad-13 was observed in PGY medium without glucose supplementation. Accordingly to Subagiyo *et al.*, (2015), carbohydrate is the main sources of carbon and energy for growth. Peptone, yeast extract and glucose were the necessary components for cell mass production and glucose is the most preferred carbon source for bacterial strains. Since PGY medium contains glucose, peptone and yeast extract which were the main components for growth, thus PGY medium is the best medium for growing of *L. plantarum* Dad-13. Pepton as well as yeast extract is considered to be a good nitrogen and carbon sources (Elsayed *et al*., 2014). PGY medium with peptone eliminated produced significantly higher growth than that glucose was removed due to the existence of yeast extract as a nitrogen source in the medium while glucose is the main carbon sources for growth.
Production of intracellular uricase by *L. plantarum* Dad-13 was affected by nitrogen, carbon sources and uric acid in the medium (Figure 2).

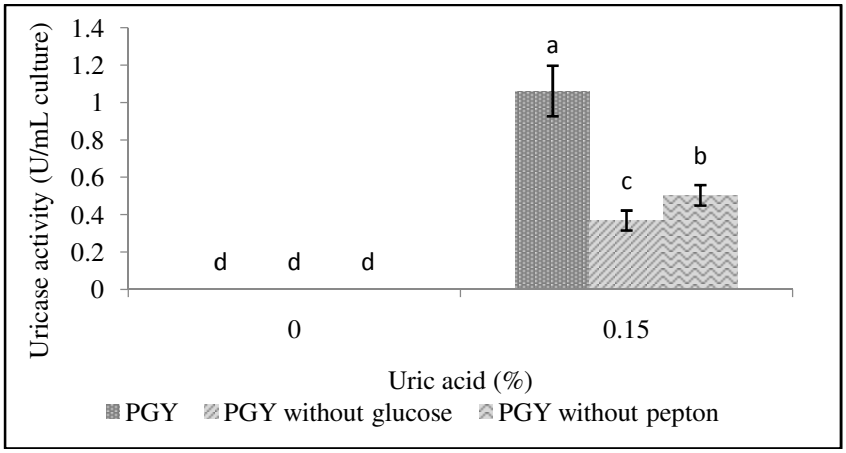

Figure 2: The effect of nitrogen compound and glucose in the PGY medium on uricase activity after fermentation by L. plantarum Dad-13 at 37°C for 22 h.

Result of this research showed that the highest uricase production was reached when the cell grown in the PGY medium but the lowest of that was resulted in the PGY medium without pepton supplementation as nitrogen sources. This result was in accordance with the intracelluler of uric acid concentration. It suggested that synthesis of uricase is affected by intracellular of uricase. Since uric acid plays important role to induce uricase synthesis, the lowest uricase activity occured in the cell when grown in the PGY medium without peptone supplementation. Alamillo *et al*., (1998) reported that synthesis of uricase is controled at the transcriptional level and its required of uric acid. Thus, intracellular uricase produced by *L. plantarum* Dad-13 was categorized as an indusible enzyme. Furthermore, Nanda *et al*., (2012); Ali and Ibrahim (2013), observed that peptone increased the uricase activity in *Gliocladium viridae* and acted as the best nitrogen source for uricase production by *Aspergillus niger*.

Since uric acid played the crucial role in forming an active uricase by *L. plantarum* Dad-13, thus in order to uricase induction, the high quantity of uric acid in the cell was needed for effective induction of uricase. Watanabe and Ohe (1973) reported that for effective induction of intracellular uricase, the high quantity of the uric acid was required for induction of intracellular uricase.
The result of this research, based on Figure 3 showed that nitrogen compound, glucose source and uric acid concentration had significant effect on the intracellular of uric acid.

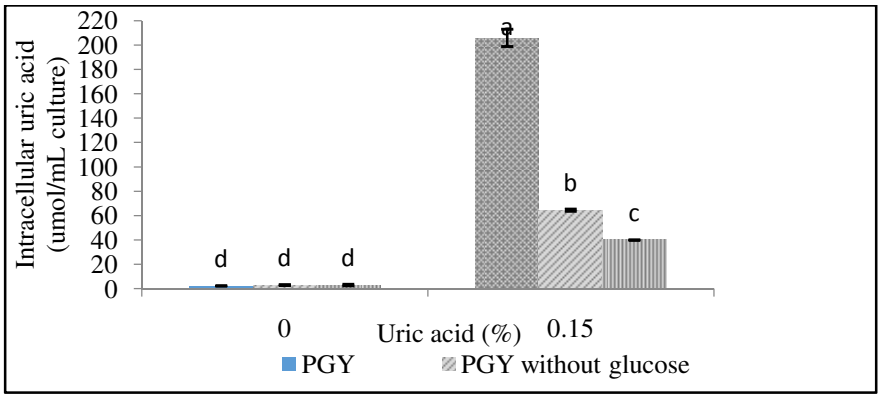

Figure 3: The effect of nitrogen compound and glucose in the PGY medium on intracellular of uric acid after fermentation by L. plantarum Dad-13 at 37°C for 22 h.

The highest concentration of intracellular uric acid resulted when *L. plantarum* Dad-13 was grown in the PGY medium while the lowest of that was produced in the PGY medium without pepton supplementation. It suggested that pepton have significantly effect on the transportation of uric acid into the cell. Pineda and Jacobo (1985) stated that uric acid transportation system debased on the novo protein sythesis. Furthermore, Watanabe and Ohe (1973) reported that the entering of uric acid into the cell is caused by the gradient concentration of uric acid inside and outside of the cell. Therefore, the enhancement of uric acid concentration in the medium, can enhance the uptake of uric acid into the cell resulting uricase formation by cells.
After fermentation, pH changed into acidic due to glucose metabolism toward lactate production. Nitrogen compound and glucose had the significant effect on pH after fermentation (Figure 4).

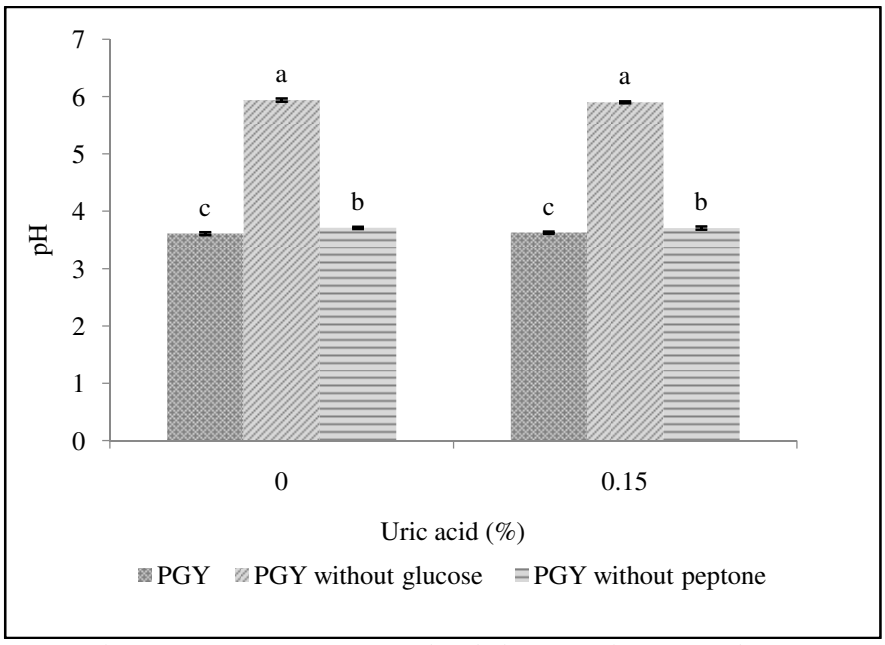

Figure 4: The effect of nitrogen compound and glucose in the PGY medium on pH after fermentation by L. plantarum Dad-13 at 37°C for 22 h.

The lowest pH was reached from the PGY medium due to glucose which was fermented by the highest amount of cell in PGY medium since PGY is the best medium for growing of *L. plantarum* Dad-13 than the other medium in this research. On the other hand, uric acid supplementation did not significantly affect the pH of medium since uric acid was categorized as a weak acid which produces allantoin during its catabolism. The addition of uric acid, thus, did not contribute significant effect.

4. Conclusion

Uricase is an inducible enzyme produced by *L. plantarum* Dad-13. Uric acid is the best inducer for uricase production while adenine and guanine can not induced this enzyme. The highest uricase activity was produced in the PGY medium when intracellular uric acid at the highest concentration while the lowest of uricase as well as intracellular of uric acid resulted in the PGY medium with peptone were omitled due to low transfered of uric acid into the cell.

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6. Author Disclosure Statement

The authors declare no conflict of interest.

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