

Screening of lactic acid bacteria producing uricase and stability assessment in simulated gastrointestinal conditions

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Article history

Received: 11 April 2017

Received in revised form:

24 May 2017

Accepted: 25 May 2017

Abstract

Thirteen probiotic lactic acid bacterial strains were screened for extracellular and intracellular uricase production. Results showed that all the strains could produce uricase with three strains, namely *Lactobacillus* sp.OL-5, *Lactobacillus plantarum* Mut-7, and *Lactobacillus plantarum* Dad-13, producing high intracellular uricase activities. Further analysis in simulated gastrointestinal conditions showed that *Lactobacillus* sp. OL-5, and *Lactobacillus plantarum* Dad-13 intracellular uricase, remained active after addition of gastric juice and duodenal juice. On the other hand, the intracellular of *Lactobacillus plantarum* Mut-7, and as well as both extracellular and membrane bound uricases of the three strains were not stable in gastric juice and duodenal juice. Thus based on uricase production and the stability in simulated gastrointestinal system, *Lactobacillus plantarum* Dad-13 is a potential strain for producing uricase.

Keywords

Lactic acid bacteria

Stability

Gastrointestinal system

Uricase

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Introduction

Uricase (E.C.1.7.3.3, urate oxidase, urate oxygen oxidoreductase) is an enzyme in the purine breakdown pathway that catalyzes the oxidation of uric acid in the presence of oxygen into allantoin and hydrogen peroxide through a complex reaction mechanism (Imhoff *et al.*, 2003; Gabison *et al.*, 2008). Allantoin is more water soluble than uric acid, so, it was readily excreted in urine (Ganson *et al.*, 2006; Sherman *et al.*, 2008; Khade and Srivastava, 2015).

Uricase has been used in medicine and clinical biochemistry as a diagnostic reagent for the measurement of uric acid in blood and other biological fluids. Iswantini *et al.*, (2016) used uricase produced by *Lactobacillus plantarum*, depositing within biofilm and planktonic bacteria on glassy carbon electrode (GCEb and GCE) using as uric acid biosensor for longer life time of this bacteria. Uricase could also be used as protein drug for hyperuricemia treatment. Hyperuricemia is a disease, result by abnormally high level of uric acid in the blood, which cause from the overproduction and/or underexcretion of uric acid and is greatly influenced by a high dietary intake of purine (Ogawa, 2006; Li *et al.*, 2014). Reduction the level of uric acid in blood and tissues is important to prohibit and treat many uric acid-related diseases (Cheng *et al.*, 2012).

Li *et al.* (2014) reported that application lactic acid bacteria strain DM9218-A to the hyperuricemic rat significantly decreased uric acid level.

Some microorganisms that were found to produce uricase are *Pseudomonas aeruginosa* (Saeed *et al.*, 2004), *Streptomyces graminofaciens*, *S. albidoflavus* and *Proteus vulgaris* (Azab *et al.*, 2005), *Microbacterium* (Zhou *et al.*, 2005), *Bacillus thermocatenulatus* (Lotfy, 2008), *Lactobacillus plantarum* (Iswantini *et al.*, 2009), and *Bacillus cereus* (Amirthanathan and Subramaniyan, 2012). *P. aeruginosa*, *B. thermocatenulatus* and *B. subtilis* were found to produce extracellular uricase (Saeed *et al.*, 2004; Lotfy, 2008; Amirthanathan and Subramaniyan, 2012) while *P. vulgaris*, *S. graminofaciens*, *S. Albidoflavus* (Azab *et al.*, 2005), and *Microbacterium* (Zhou *et al.*, 2005) were found to produce intracellular uricase. The intracellular uricase is more stable in gastrointestinal system (O'Connell and Walsh, 2007). Uricase production was induced by uric acid in the growth media. Uricase formation might be controlled by a metabolite repression mechanism, in which a metabolite derived from both the nitrogen and carbon sources may participate (Rouf and Lompfrey, 1968; Bongaerts and Vogels, 1976; Nahm and Marzluf, 1987).

Li *et al.*, (2014) screened fifty-five lactic acid bacteria isolated from chinese sauerkraut, which

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one of them, namely DM9219-A has potential as a probiotic in the prevention of hyperuricemia. On the other hand, some local isolates of lactic acid bacteria have shown probiotic properties (Ngatirah *et al.*, 2000; Purwandani *et al.*, 2000; Rahayu *et al.*, 2011). Iswantini *et al.* (2009) also reported on lactic acid bacteria-producing uricase, such as *L. plantarum*. Therefore, screening of probiotic lactic acid bacteria for producing uricase shall have an advantage both as probiotic and uricase sources. Since uricase may not be stable in acid condition in gastrointestinal system, uricase stability in simulated gastrointestinal conditions was used as screening criteria. The objective of this research was to obtain probiotic lactic acid bacteria, that produces uricase which remains active in gastrointestinal system. Subsequently the effect of glucose and type of media on uricase production of selected strain was evaluated.

Materials and Methods

Strains of lactic acid bacteria

Lactobacillus plantarum Dad-13, *L. plantarum* Mut-7, *L. acidophilus* SNP-2 and *L. plantarum* T-3 were obtained from Food and Nutrition Culture Collection (FNCC), Center for Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta. In addition, *Lactobacillus* sp. OL-2, *Lactobacillus* sp. OL-3 *Lactobacillus* sp. OL-5, *Lactobacillus* sp. MS-12, *Lactobacillus* sp. MS-15 and *Lactobacillus* sp. OT-16 were obtained from the Laboratory of Microbiology, Department of Agricultural Technology, Faculty of Agriculture, Universitas Jenderal Soedirman, Central Java. Isolates were 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 stock culture.

Preparation of starter culture

The strain was cultivated by adding 0.1 mL of stock culture in 10 mL of Peptone Glucose Yeast Extract (PGY) medium containing glucose 10 g, yeast extract 10 g, peptone 5 g, Na-asetat 1.4 g, beef extract 2 g, tween 80 10 ml, and salt solution 5 ml in 1 L (Iswantini *et al.*, 2009). It was incubated at 37°C for 18 h. The cultivation of the culture was done twice.

Screening of uricase producing lactic acid bacteria

Thirteen probiotic lactic acid bacteria strains, which produced uricase, were screened by both well diffusion agar method and fermentation in PGY medium containing uric acid (Iswantini *et*

al., 2009). PGY agar medium and broth medium were supplemented with uric acid 0.5% and 0.2% as inducer, respectively. The well diffusion assay was carried out by adding 50 µL culture starter of bacterial strain into wells (8 mm diameter). It was incubated at 37°C for 5 days. Subsequently, the strains, which had no clear zone and clear zone diameter of about 0.2 cm were further incubated in PGY broth medium containing 0.2 % uric acid at 37°C for 24 h. The uric acid residues were further determined. Four (4) isolates with the highest clear zone which indicated produced high extracellular uricase and four (4) isolates with 0.2 cm and no clear zone which highest decreasing uric acid were chosen to evaluate the activities of extracellular, intracellular and membrane bond uricase as selected strains.

Extracellular, intracellular and membrane bond uricase activities.

About 1% culture starter of the selected lactic acid bacteria strains were inoculated in PGY broth medium containing 0.2% of uric acid. They were incubated at 37°C for 24 h. Subsequently, the culture was centrifuged at 3000 rpm for 20 min at 4°C to separate supernatant (crude extracellular uricase) from the bacterial cell. The cell was further washed twice with 5 mL 0.1 M sodium phosphate buffer (pH 7.0) (Kai *et al.*, 2007). Disruption of cells were done by adding quartz sand (150-212 µm) to the cell suspension and stirring vigorously for 10 min, with occasional cooling in the ice bath (Carevic *et al.*, 2015). The cell debris were separated by centrifugation at 6000 rpm for 20 min at 4°C. The supernatant was removed and used as crude intracellular uricase. About 1 mL of 1% Triton X 100 and 2 mL of 0.1 M phosphate buffer (pH 7.0) was added into the natant. It was then centrifuged at 6000 rpm, for 20 min, at 4°C. Supernatant was used as crude membrane bond uricase. Extracellular, intracellular and membrane bond were further stored at -40°C until use.

The stability of the extracellular uricase in stomach model

Uricase stability on stomach was determine according to Hur *et al.* (2011) with some modifications. The simulated gastric juice (12 mL, pH 2) was added into 5 mL of crude extracellular uricase. The mixture was then incubated at a shaker incubator (Model HB-205SW, Hanbaek, Co., Bucheon, Korea) at 37°C for 30 min. It was further centrifuged at 3000 rpm for 20 min at 4°C. Then the residue of uricase activity was measured.

The stability of extracellular uricase in small intestine model

Uricase stability in small intestine was determined according to Hur *et al.* (2011) with some modifications. About 12 mL of duodenal juice, 6 mL of bile juice and 2 mL of HCO₃ solution (pH 6.5-7.0) were added into 5 mL of the remaining uricase from gastric juice test. The mixture was then incubated at 37°C for 30 min. The supernatant was centrifuged at 3000 rpm for 20 min at 4°C. The remaining uricase activity was then measured.

The stability of intracellular and membrane bound uricase in stomach model

The simulated gastric juice (12 mL, pH 2) was added into bacterial cell, then the mixture was incubated in a shaker incubator (Model HB-205SW, Hanbaek, Co., Bucheon, Korea) for 30 min at 37°C. Furthermore the cells were separated by centrifugation at 3000 rpm for 20 min at 4°C. They were further washed twice with 0.1 M sodium phosphate buffer (pH 7.0). The intracellular and membrane bound uricase were obtained by disrupting the cells using quartz sand method. Uricase activities were further determined.

The stability of intracellular and membrane bound uricase in small intestine model

The stability of intracellular and membrane bound uricase in small intestine were determined according to Hur *et al.* (2011) with some modifications. About 12 mL of duodenal juice, 6 mL of bile juice and 2 mL of HCO₃ solution (pH 6.5-7.0) were added into the remaining bacterial cell uricase from gastric juice test. The mixture was incubated for 30 min at 37°C. The simulated juice was separated by centrifugation at 3000 rpm for 20 min at 4°C. The bacterial cells were disrupted. After that the intracellular and membrane bound uricase activity were determined. The strains, which produced stable uricase from the gastrointestinal system test, were selected and evaluated for the next step.

Effect of media on uricase activity

The selected strain was grown in 45 mL of MRS (Ogawa, 2006) and PGY medium that was supplemented with 0.15% uric acid. It was incubated at 37°C for 22 h. MRS medium was supplied by name of supplier/manufacturer (Iswantini *et al.*, 2009).

Uricase production at various glucose concentration of selected strain

The selected strain was incubated in 45 mL of media containing various glucose concentrations

(0%, 1%, 3%, 5%, 7% and 9%) containing 0.15% uric acid. It was incubated at 37°C for 22 h. Glucose residue and cell number were determined by Nelson Somogyi method and total plate count, respectively.

Uricase assay

Uricase activity was determined according to Bergmeyer (1974) with a modification. About 0.08 mL of crude uricase was added into a mixture of 3.08 mL of borate buffer pH 8.0 and 0.01 mL 3.57 mM uric acid and was incubated for 10 min at 37°C. The reaction was stopped by boiling the mixture at 5 min. Beside, 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 equivalent to the amount of enzyme, which convert 1 μmol of uric acid to allantoin per min at 37°C.

Results and Discussion

Lactic acid bacteria strains producing extracellular uricase

The microorganisms producing extracellular uricase converted the suspended uric acid in the agar medium into water soluble allantoin, which developed a clear zone around the well (Dwivedi *et al.*, 2012). This method is a novel, simple, inexpensive and sensitive technique for screening of microbes for uricase production and estimation of uricase production by some microbes (Dwivedi *et al.*, 2012). The diameter of the developed clear zone is directly related to extracellular uricase activity i.e a bigger clear zone diameter indicated that extracellular uricase activity was higher. About 10 strains developed a clear zone in the well diffusion agar method. Diameter of clear zones was in the range 0.2-1.4 cm after 5 days of incubation (Table 1). *Lactobacillus acidophilus* SNP-2 produced the largest clear zone. Thus, it indicated that those strains produced extracellular uricase enzyme. On the other hand, three strains, namely *Lactobacillus* sp OL-2, *Lactobacillus* sp OL-3 and *Lactobacillus* sp OL-5, did not have clear zone. It indicated that they did not produce extracellular uricase.

Strains, which have a clear zone diameter from 0 to 0.2 cm were evaluated for intracellular uricase activity in PGY broth medium containing uric acid. The result shows that uric acid in the medium decreased after 24 h (Table 1). It is indicated that all strains produced uricase. It is suggested that three

Table 1. Screening of the produced uricase strains using agar method and fermentation in PGY medium contain uric acid

Strains	Diameter of clear zones (cm)	Decrease of uric acid in the medium (mg/mL)
<i>Lactobacillus sp</i> OL-2	0	1.60 ± 0.1
<i>Lactobacillus sp</i> OL-3	0	1.67±0.1
<i>Lactobacillus sp</i> OL-5	0	1.68±0.1
<i>Lactobacillus sp</i> MS-12	1.3±0.10	Not analyzed
<i>Lactobacillus sp</i> MS-13	0.5±0.1	Not analyzed
<i>Lactobacillus sp</i> MS-14	0.6±0.2	Not analyzed
<i>Lactobacillus sp</i> MS-15	1.3±0.2	Not analyzed
<i>Lactobacillus sp</i> OT-16	0.9±0.1	Not analyzed
<i>Lactobacillus plantarum</i> Mut-7	1.0±0.0	Not analyzed
<i>Lactobacillus plantarum</i> Mut-13	0.7±0.1	Not analyzed
<i>Lactobacillus plantarum</i> Dad-13	0.2±0.0	1.73±0.12
<i>Lactobacillus acidophilus</i> SNP-2	1.4±0.4	Not analyzed
<i>Lactobacillus plantarum</i> T-3	0.2±0.0	1.59±0.08

strains, i.e. *Lactobacillus sp* OL-2, *Lactobacillus sp* OL-3, and *Lactobacillus sp* OL-5, produced only intracellular uricase, since they could reduce uric acid in the medium, but they could not produce a clear zone in agar medium. Iswantini *et al.* (2009) reported that *L. plantarum* K. Mar. E, *L. plantarum* Mgs Psmb and *L. plantarum* Mgs. Bst with 0.2 cm in diameter of clear zones have uricase activities. Cheng *et al.* (2012) reported that crude recombinant lactic acid bacteria with urate oxidase was slightly lower. Thus *Lactobacillus sp.* OL-2, *Lactobacillus sp.* OL-3, *Lactobacillus sp.* OL-5, *Lactobacillus sp.* MS-12, *Lactobacillus sp.* MS-15, *Lactobacillus plantarum* Mut-7, *Lactobacillus plantarum* Dad-13 and *Lactobacillus acidophilus* strain SNP-2 were selected for further evaluation on uricase production.

Uricase activities of the selected strains

The activities of extracellular, intracellular and membrane bound uricase of the selected strains are shown in Figure 1. All strains produced extracellular, intracellular and membrane bound uricases. In addition, the extracellular uricase was mostly produced compared with intracellular and membrane bound uricase. Clear zone has a correlation with producing extracellular uricase. *Lactobacillus plantarum* SNP-2, which had the highest clear zone, had also the highest extracellular uricase (1.54 U/ml culture). On the other hand, *Lactobacillus sp.* OL-2, *Lactobacillus sp.* OL-3 and *Lactobacillus sp.* OL-5, which had no clear zone, had low extracellular uricase activity, namely about 0.37; 0.59 and 0.59 U/mL culture, respectively. However, *Lactobacillus plantarum* Dad-13, which had lower clear zone and extracellular

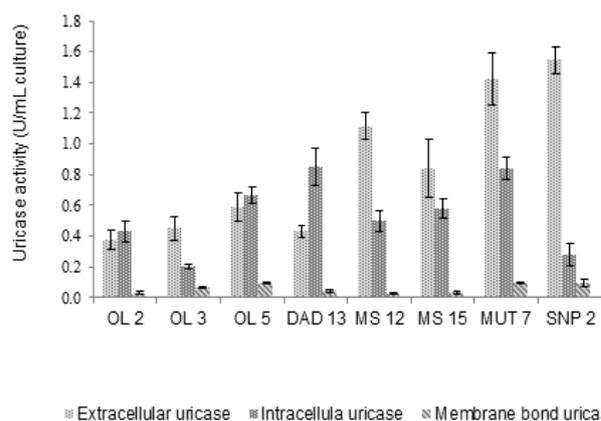


Figure 1. Extracellular, intracellular and membrane bound uricase activity from various lactic acid bacterial strains. Fermentation was performed in PGY medium supplemented with 0.2% of uric acid. It was incubated for 24 h at 37°C.

uricase, had the highest intracellular uricase (0.85 U/mL culture). Furthermore, the stability of uricase in gastrointestinal system from three strains, which had high intracellular activities (*Lactobacillus sp.* strain OL-5, *Lactobacillus plantarum* Mut-7, *Lactobacillus plantarum* Dad-13) were further evaluated.

Uricase stability and activity in gastrointestinal system

The result shows that the activities of extracellular and membrane bound uricase of *Lactobacillus sp.* OL-5, *Lactobacillus plantarum* Dad-13 and *Lactobacillus plantarum* Mut-7 were not detected after addition of gastric juice (Fig. 2A and 2C). Uricase is the homotetrameric enzyme, in which the active site is located at the interface of two symmetric monomers (Colloc'h *et al.*, 2006). Gabison *et al.* (2008) suggested that amino acid residue of the uricase active site was in the form of phenylalanine 159 close to the substrate bonds site. At low pH, polypeptide changes to unfolding form making the enzyme in-active. On the other hand, the presence of pancreatic enzyme was able to hydrolyze the active site of uricase of the peptide bonds, namely the residues of arginine, lysine and aromatic amino acid (Gabison *et al.*, 2008; Fuhrmann and Leroux, 2014).

The intracellular uricase of *Lactobacillus sp.* OL-5 as well as *Lactobacillus plantarum* Dad-13 remained active in the gastrointestinal system (Figure 2B). The remaining uricase activity of *Lactobacillus sp.* OL-5 and *Lactobacillus plantarum* Dad-13 after addition of gastric juice were 14.24% and 16.77%, respectively. However, intracellular uricase activity of *Lactobacillus plantarum* Mut-7 was not detected. It implied that the cell membrane and cell wall maintained the intracellular cytosolic pH and also protected the uricase from the hydrolytic activity of

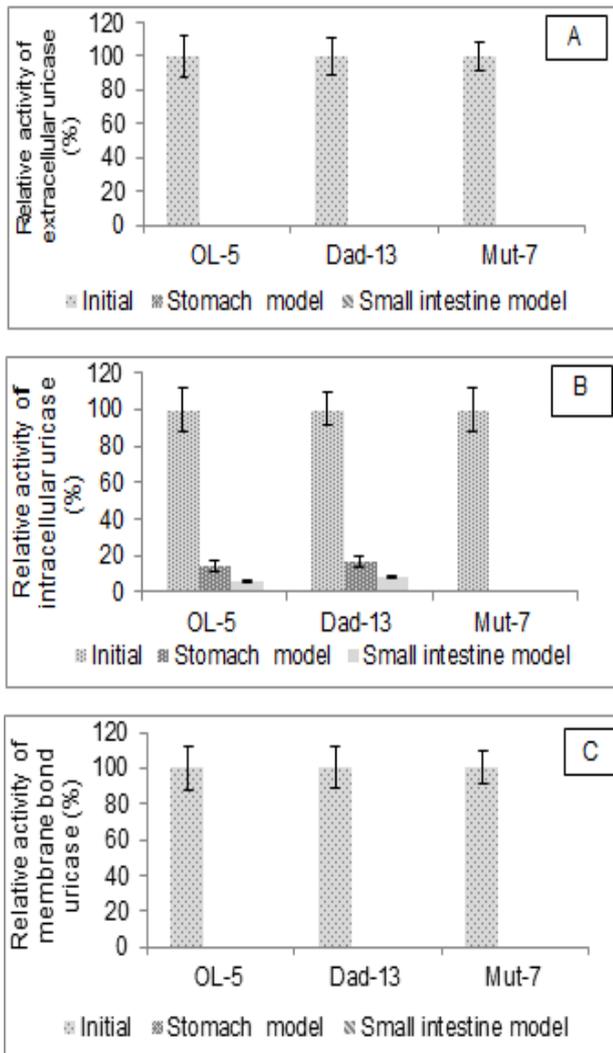


Figure 2. Stability of extracellular uricase (A), intracellular uricase (B) and membrane bound uricase (C) from various lactic acid bacteria strains in gastric juice and duodenal juice. Fermentation was performed in PGY medium supplemented with 0.2% of uric acid. It was incubated for 24 h at 37°C.

gastric protease.

On the other hand, *Lactobacillus* sp OL-5, *Lactobacillus plantarum* Dad-13 and *Lactobacillus plantarum* Mut-7 were probiotic lactic acid bacteria (Ngatirah *et al.*, 2000; Sujiman *et al.*, 2014). Therefore, they were tolerant to adverse conditions in the gastrointestinal system. The differences in acid tolerance of some species of lactic acid bacteria was associated with the selective permeability of proton (Cotter and Hill, 2003).

Uricase stability and activity in duodenal juice system

The intracellular uricase activity of *Lactobacillus* sp. OL-5 and *Lactobacillus plantarum* Dad-13 remained active after addition of gastric juice. The remaining activities after addition of duodenal juice were 5.95% and 8.28%, respectively (Fig. 2B). Thus some uricase of probiotic lactic acid bacteria remained

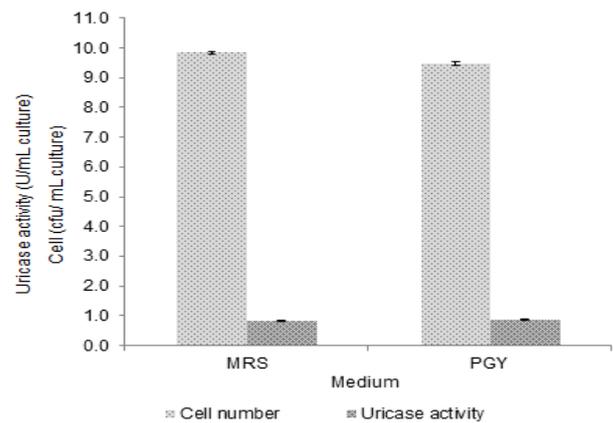


Figure 3. The effect of media on uricase production by selected strain (*Lactobacillus plantarum* Dad-13). MRS and PGY medium was supplemented with 0.15% of uric acid. It was incubated at 37°C for 22 h.

active in gastrointestinal system. It is suggested that they can be used as oral food for the prevention or treatment of hyperuricemia. *Lactobacillus plantarum* Dad-13 was used as the selected strain to be evaluated on further test.

Effect of medium on uricase production

Figure 3 shows that uricase activities of *Lactobacillus plantarum* Dad-13 in MRS and PGY medium was not significantly different. But the cell numbers were significantly different. MRS and PGY medium were the basal medium for lactic acid bacteria, which supported the growth of cells. MRS medium contains glucose and peptone higher than PGY medium. Therefore, the cell growth in MRS medium was higher than that in PGY medium. However, the uric acid in MRS and PGY medium were quite similar. Therefore, the produced uricase in the two media were not significantly different.

The effect of glucose concentration on uricase activity

Figure 4 shows that uricase activity increased with an increase in glucose concentration from 0 to 1%. Further increase in glucose concentration to 3% resulted in a decrease in uricase activity. Uricase was produced under repression condition. High glucose concentration, which was higher than 3%, had a negative effect on uricase production. It is suggested that high glucose concentration inhibited uricase production of *Lactobacillus plantarum* Dad-13. This result was in agreement with Nahm and Marzluf (1987) and Nanda *et al.* (2012). An increase in glucose concentration from 0 to 5% resulted in an increase in cell during incubation. Further increase in glucose concentration did not have a significant effect on the cell number due to the decrease in water activity. Therefore, the remaining glucose residue in the media increased with an increase in glucose

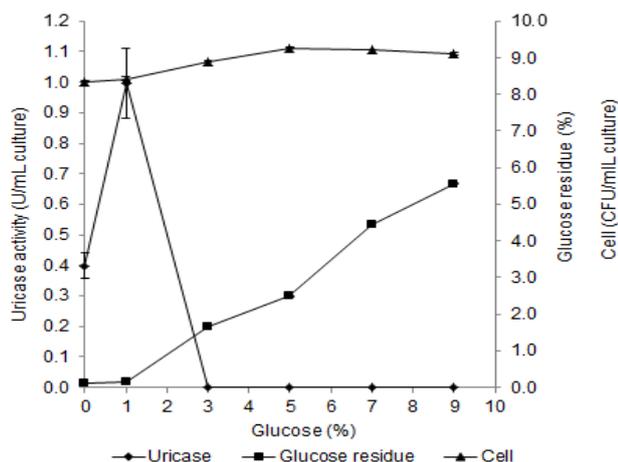


Figure 4. The effect of glucose concentration on uricase production of the selected strain (*Lactobacillus plantarum* Dad-13). Fermentation was performed in PGY medium supplemented with 0.15% of uric acid. It was incubated at 37°C for 22 h.

concentration.

Conclusion

All the probiotic strains produced uricase. *Lactobacillus* sp. OL-5, *Lactobacillus plantarum* Mut-7 and *Lactobacillus plantarum* DAD-13 produced high intracellular uricase activity. However, *Lactobacillus* sp. OL-5 and *Lactobacillus plantarum* DAD-13 produced intracellular uricase, which remained active in the gastrointestinal system. But the extracellular and membrane bound enzymes of the three isolates and intracellular of *Lactobacillus plantarum* Mut-7 were not stable in the gastrointestinal system.

Lactobacillus plantarum Dad-13 is good isolate as probiotic and uricase source, since it produced higher uricase and remained active in the simulated gastrointestinal system. Uricase production in MRS medium did not have a significant difference compared with PGY medium. On the other hand, high glucose concentration in medium inhibited uricase production.

Acknowledgement

The authors gratefully acknowledge the Directorate General of Higher Education, Ministry of Research, Technology, and Higher Education, Republic of Indonesia, for awarding the Doctoral Research Grant under which the present project was carried out.

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