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Research Article
Enhancement of an Intracellular Uricase Produce by *L. plantarum* Dad-13 Which has Stability in Gastrointestinal System

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Abstract

Background: *Lactobacillus plantarum* Dad-13 is a probiotic lactic acid bacteria strain that produces uricase for reducing uric acid. **Objective:** The aim of study was to evaluate the stability of intracellular uricase produced by *L. plantarum* Dad-13 in the gastrointestinal system and to enhance the production of this enzyme. **Materials and Methods:** *Lactobacillus plantarum* Dad-13 was grown on Peptone Glucose Yeast extract (PGY) medium supplemented with uric acid as inducer. The stability of intracellular uricase was evaluated in the stomach and small intestine models. For enhancement of uricase production, *L. plantarum* was grown on variation of incubation time, uric acid concentration and temperature, while glucose residue, intracellular uric acid were used as the criteria of evaluation. Data were statistically analyzed by one way-ANOVA followed by DMRT. **Results:** The intracellular uricase of *L. plantarum* Dad-13 remained active in the gastrointestinal system. Uricase is an inducible enzyme produced when glucose residue limited in the medium. This bacteria uptake uric acid from the medium during growth and uricase activity was obtained at the optimum concentration of uric acid in the cell. A maximum uricase activity was reached at 0.15% uric acid concentration and 37°C for 22 h of incubation. **Conclusion:** Production of intracellular uricase produced by *L. plantarum* Dad-13 which have activity in gastrointestinal tract could be enhanced by optimum fermentation and make it applicable for hyperuricemia treatment.

Key words: *Lactobacillus plantarum* Dad-13, intracellular uricase, stability, gastrointestinal tract, optimum fermentation

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Uricase that is categorized as oxidoreductase enzyme and participates in the purine breakdown pathway is a biocatalyzator for the oxidation of uric acid into allantoin and hydrogen peroxide[1,2]. Humans lacking functional uricase will eliminate the uric acid as the end product of purine catabolism. The increasing of uric acid level in blood over the normal value (hyperuricemia) can lead to gout disease. Rasburicase which is a protein drug has been used for cure of hyperuricemia[3].

Lactic acid bacteria, such as *L. plantarum* can produce uricase[4]. *Lactobacillus plantarum* Dad-13 is lactic acid bacteria that has the ability as probiotic source[5]. 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. But orally-administration uricase for treatment hyperuricemia remains challenging. Low pH, pepsin, pancreatic and bile salt in gastrointestinal tract induced protein unfolding and propensity cleaved peptide bond resulting of enzyme inactivation[6]. Amylase, lipase, protease and cellulase were exogenous enzymes which were produced by probiotics, have an ability to help endogenous enzymes in hydrolysing of nutrients[7]. Sacrosidase which is used for the treatment of congenital sucrose-isomaltase intolerance has stability in gastrointestinal system[6]. Therefore, evaluation the stability of intracellular uricase produced by *L. plantarum* of Dad-13 in the gastrointestinal system had an advantage both as probiotic and uricase producer.

The production of uricase by some microorganisms such as *Mucor hiemalis*, *Bacillus cereus*, *Xanthomonas fuscans* and *Bacillus substilis* were affected by some fermentation conditions such as incubation time, uric acid concentration and temperature[8-11]. Purine and purine derivates can used as inducers for uricase activity and uric acid are the best inducer by some microorganism[12]. Uricase production was controlled by a metabolite repression of nitrogen and carbon source[11].

In order to obtain higher activity of uricase for reduction of uric acid, the present investigation aimed to enhance the production of intracellular uricase produced by *L. plantarum* Dad-13 that has an activity in the gastrointestinal system.

MATERIALS AND METHODS

Strains: *Lactobacillus plantarum* Dad-13, which was isolated from fermented milk, was obtained from Food and Nutrition Culture Collection (FNCC), Centre for Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta, Indonesia, was used for uricase production. Strain was stored in 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.

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.

Production of intracellular uricase: A 1% culture starter of *L. plantarum* Dad-13 was inoculated in PGY medium broth containing 0.2% of uric acid. Incubation was done at 37°C for 24 h for uricase production. After fermentation, the culture was centrifuged at 3000 rpm at 4°C for 20 min to separate the supernatant. Then the bacterial cell was used to evaluate the stability of intracellular uricase in the stomach and small intestine models.

Stability of intracellular uricase in stomach model: Uricase stability on stomach and small intestine were determined according to Hur *et al.*[13] with some modifications. 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) at 37°C for 30 min. Furthermore, centrifugation at 3000 rpm for 20 min at 4°C was done to separated the supernatant. The cell was washed twice with 0.1 M sodium phosphate buffer (pH 7.0), followed by preparation and examination of uricase.

Preparation of intracellular uricase: The 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[14]. 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[15]. To separate the cell debris, the sample was centrifuged at 6000 rpm (4°C) for 20 min and the supernatant used as crude intracellular uricase then uricase was assayed.

Stability of intracellular uricase in small intestine model: The bacterial cells after washing in gastric juice test were added by 12 mL of duodenal juice, 6 mL of bile juice and 2 mL of HCO_3 solution (pH 6.5-7.0)[13]. The mixture was incubated at 37°C for 30 min. The simulated juice was separated by centrifugation at 3000 rpm (4°C) for 20 min, follow by preparation and determination of intracellular uricase.

Incubation time for uricase production: The time-course of uricase production was studied using *L. plantarum* Dad-13 which was grown in the 700 mL PGY media in 1 L Erlenmeyer consists of 10 g glucose, 10 g yeast extract, 5 g peptone, 1.4 g Na-acetate, 2 g beef extract, 10 mL Tween 80 and 5 ml L^{-1} salt solution media, at 37°C for 24 h. A 0.2% of uric acid was added to the media and the growth was allowed to continue. A 40 mL media were harvested every 2 h.

Uric acid concentration: To find out the optimum concentration of inducer for uricase production by *L. plantarum* Dad-13, the culture media was adjusted with different concentrations of uric acid used as inducers i.e. 0, 0.5, 0.10, 0.15 and 0.2% (g v^{-1}). *Lactobacillus plantarum* Dad-13 was grown in 45 mL of PGY medium induced with different concentrations of uric acid in the achieved optimum conditions of the incubation time.

Temperature: To study the effect of temperature for uricase production, *L. plantarum* Dad-13 was grown in 45 mL of PGY medium at various incubation temperatures, i.e., 20, 30, 37 and 42°C which represent lower mesophilic, room, human body and upper mesophilic temperatures, respectively, in the achieved optimum conditions of the incubation period and uric acid concentration.

Cell, glucose residue and uric acid enumeration: The effect of incubations time, uric acid concentrations and temperatures on the growth of *L. plantarum* Dad-13, glucose residue and intracellular of uric acid were carried out as follows. The growth of cells were determined by pour plate count method. Glucose residue was determined by Nelson Somogyi method and intracellular of uric acid was determine by spectrophotometer at 293 nm[16].

Uricase assay: Uricase activity was assayed according to Iswantini *et al.*[4] with some modifications. A 0.08 mL of crude intracellular uricase was added into a mixture of 3.09 mL borate buffer pH 8.0 and 0.01 mL 3.57 mM uric acid. The mixture was incubated at 37°C for 10 min. The reaction was stopped by boiling the mixture at 5 min. As a reference, mixture was boiled directly after addition of crude intracellular uricase. 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 convert 1 µmol of uric acid to allantoin per min at 37°C.

Statistical analysis: Statistical analysis were performed using one-way analysis of variance (ANOVA) followed by Duncan Multiple Range Test (DMRT) for comparison of several means. Values of $p<0.05$ were considered significant[17].

RESULTS

Stability of intracellular uricase in stomach and small intestine: The additional of gastric juice uricase and duodenal juice resulted in decreasing of uricase activity and the presence of gastric juice gave the highest effect on uricase activity (Fig. 1). The intracellular uricase activity was 0.71 U mL^{-1} culture at initial of evaluation. The remaining of uricase activity was 0.11 U mL^{-1} culture after gastric juice test and 0.06 U mL^{-1} culture after duodenal juice test. Since *L. plantarum* Dad-13 produced intracellular uricase which still have activity in gastrointestinal system, so further evaluation to enhance of uricase production was conducted.

Incubation time: *Lactobacillus plantarum* Dad-13 started the exponential phase of growth after the 2 h of the time of incubation and reached the stationary phase at 12 h of incubation. Uricase was produced by *L. plantarum* Dad-13 in the stationery phase after the 16 h of incubation, when glucose residue limited (0.22%) in the medium. The optimum uricase produced by *L. plantarum* Dad-13 was reached at 22 h and started declining at 24 h of incubation (Fig. 2).

During incubation, *L. plantarum* Dad-13 accumulated the uric acid in the cell (Fig. 3). The accumulation of uric acid in the cell was in line with the growing of the cell.

Uric acid concentration: Uric acid concentrations had no effect on the growth of *L. plantarum* Dad-13 and on the glucose residue in the medium, while uricase was not

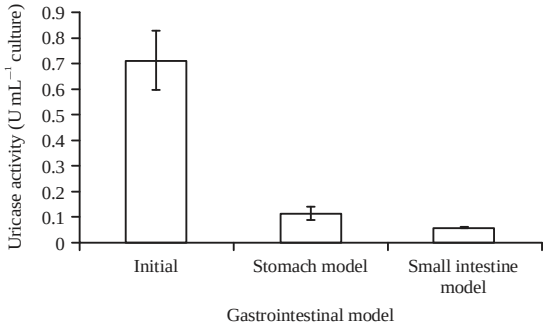

Fig. 1: Uricase stability produced by *L. plantarum* Dad-13 in stomach and small intestine models
Bars represent mean values±standard deviation in triplicate

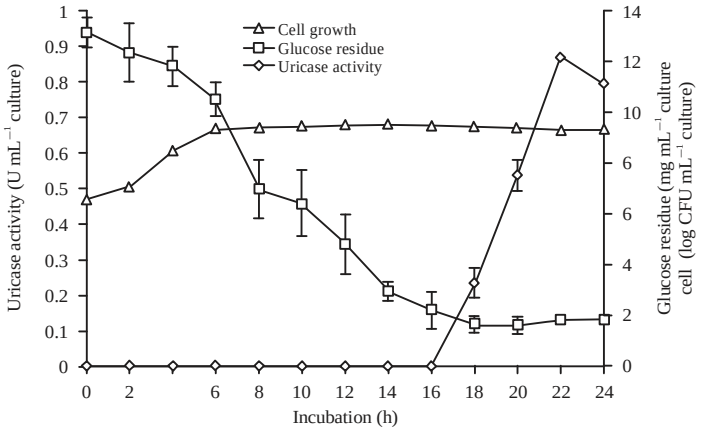

Fig. 2: Cell growth, glucose residue and uricase activity during incubation

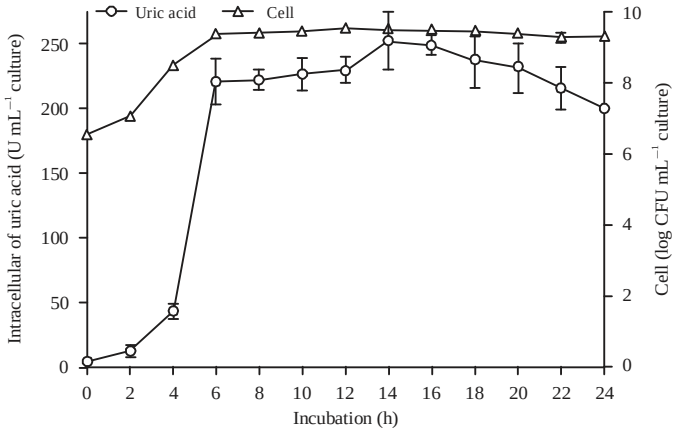

Fig. 3: Intracellular of uric acid and cell during incubation

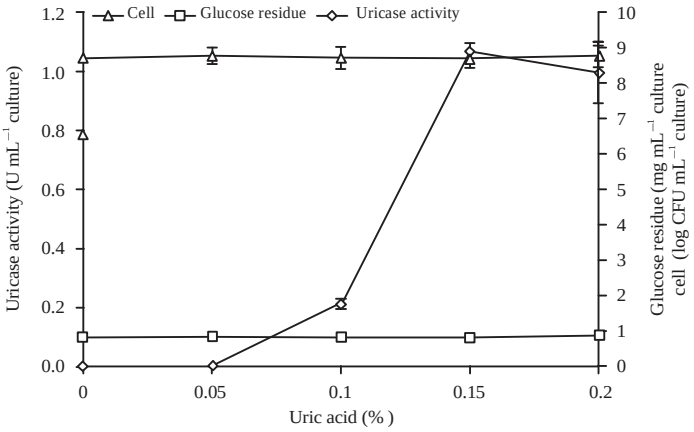

Fig. 4: Effect of concentration of uric acid on the cell, glucose residue and uricase activity

produced in medium without and with 0.05% of uric acid (Fig. 4). The unproduced uricase happened because of less uric acid in the cell. Therefore, uricase of *L. plantarum* Dad-13 was an inducible enzyme and uric acid played crucial role in forming an active uricase. The optimum uricase activity reached when 0.15% uric acid supplemented in the medium.

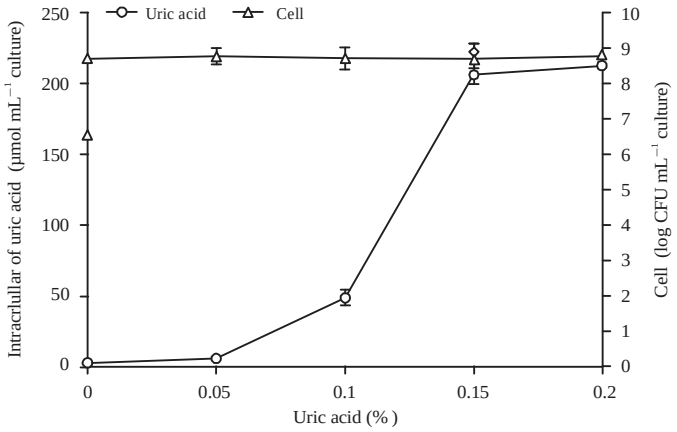

Fig. 5: Effect of uric acid concentration on the intracellular of uric acid and the cell

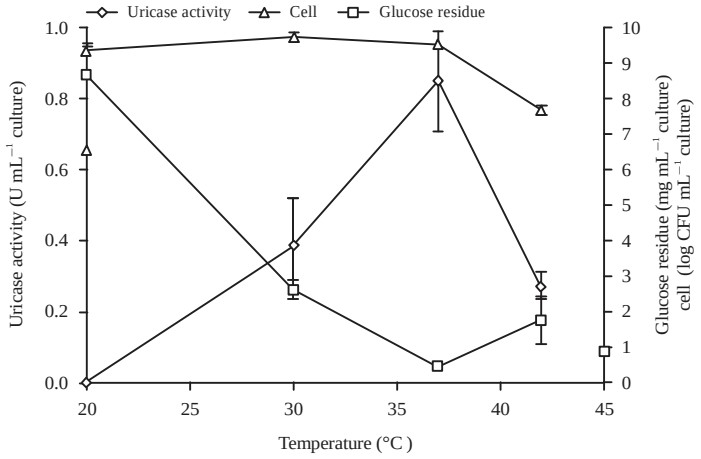

Fig. 6: Effect of temperature on the uricase activity, cell and glucose residue

The raising of uric acid concentration in the medium up to 0.15% enhanced intracellular uric acid, however with the addition of 0.2% had no significant effect (Fig. 5). This result was similar to the uricase activity when several of uric acid concentrations were supplemented into the medium.

Temperature: The optimum temperatures for growth and intracellular of uric acid by *L. plantarum* Dad-13 reached at 30°C (Fig. 6). Uricase cannot be produced when this strain grows at 20°C. At this temperature, uric acid in the cell had the lowest concentration while glucose residue had the highest concentration in the medium. Uricase production increased along with increasing temperature up to 37°C and then decreased at 42°C. The highest uric acid in the cell was established when the cell grows at 30°C since the highest uptake of uric acid occurs at this temperature (Fig. 7).

DISCUSSION

The presence of gastric juice had a great effect on decreasing of uricase activity. Since the active site of uricase located at the interface of two symmetric monomers, amino acid residues that keep the active site of uricase, in the low pH lead to unfolding and make decreasing of uricase activity[18]. The pepsin in the gastric juice will also cause hydrolysis of phenylalanine that located in the active site of uricase. Although stomach is a harsh environment for uricase activity, result of this study showed that uricase still have an activity. The remaining of intracellular uricase activity in the stomach was suggested that the cell membrane and cell wall maintained the intracellular cytosolic pH and also the selective permeability of proton protected the uricase from the hydrolytic activity of gastric protease. On the other hand, *L. plantarum* Dad-13 is a probiotic lactic acid bacteria which is tolerant into gastrointestinal system[5]. The difference of acid

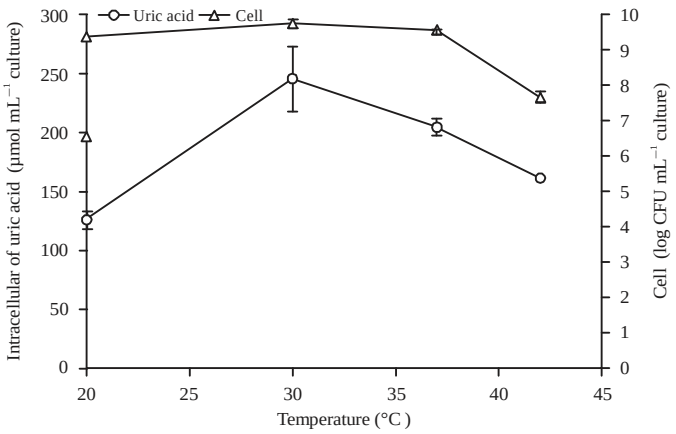

Fig. 7: Effect of temperature on the accumulation of uric acid in the cell and the cell

tolerance in some species of lactic acid bacteria is associated with selective permeability to proton. The mechanism underlying response and adaptation to low pH by lactic acid bacteria is associated with biosynthesis of fatty acid and amine accumulation to a defense mechanism to counteract acidic environment[19]. The addition of duodenal juice and bile salt will decrease the uricase activities in small intestine. Since the pH in intestinal is the optimum pH for uricase activity, the presence of pancreatic enzyme and bile salt would not cleaved all the peptide bonds of arginine, lysine and aromatic amino acid in the active site of uricase. In various lactic acid bacteria, membrane proteins mediating bile efflux from cells were the first proteins shown to be related to bile resistence[19]. Based on this research, the stability of intracellular uricase produced by *L. plantarum* Dad-13 was lower than that reported by Yuan *et al.*[20], who stated that polygalacturonase produced by *Klebsiella* sp., Y1 CGMCC 4433 under simulated alimentary tract conditions was very stable (>25% activity). Meanwhile, O'Connell and Walsh[21] reported that simulated intestinal fluid resulted in significant activity loss of galactosidase produced by *Kluyveromyces marxianus* DSM5418 over time (about 12% of residual activity was left after 2 h).

The result of the effect of incubation time on the growth of *L. plantarum* Dad-13 showed that *L. plantarum* Dad-13 reached stationary phase at 12 h of incubation. The cell, therefore, can reach stationary phase more quickly in the PGY medium than when it was growing in MRS medium due to glucose and peptone content was less in PGY medium than that in MRS medium. *Lactobacillus plantarum* Dad-13 growing in MRS medium reached stationary phase after 16 h of incubation[22].

Uricase was produced by *L. plantarum* Dad-13 at the stationery phase after the 16 h of incubation when glucose residue limited in the medium. Glucose has negative effect on uricase production. Less glucose concentration supported the higher uricase activity and uricase was produced by the cell growing in the media under repression condition[23]. This result was in accordance to Dwivedi *et al.*[24] meanwhile in contras to Ram *et al.*[10]. Dwivedi *et al.*[24] reported that there was no uricase activity during the growth of cell until the cell reached the stationary phase. While Ram *et al.*[10] reported that uricase production by *Xanthomonas fuscans* subsp *aurantifolii* increased gradually during the exponential phase. Jagathy *et al.*[11,25] reported that the incubation time to produce uricase by *Bacillus subtilis* and *A. niger* is resulted after 6 and 24 h of incubation, respectively. In this phase, due to a lack of nutrient, *L. plantarum* Dad-13 used uric acid as the source of carbon, nitrogen and energy. Aly *et al.*[12] reported that the product of uric acid oxidation by uricase is used as carbon, nitrogen and energy sources. Production of uricase by *L. plantarum* Dad-13 reached optimum at 22 h and started declining at 24 h of incubation. The reduction of uricase activity due to allantoin from uric acid oxidation will be released to the medium. Allantoin is an inhibitor for uricase[26]. During incubation, the intracellular uric acid entered and accumulated in the cell after lag phase. This result was in line with that reported by Pineda and Cardenas[27]. The entering of uric acid into the cell is caused by the gradient concentration of uric acid inside and outside of the cell. This process also needs a gradient proton. The intracellular uric acid started to decline after 16 h of incubation due to uricase was produced by the cell and oxidized of uric acid into allantoin.

The enhancement of uric acid concentration up to 0.15% increased the uricase activity but concentration at 0.2% have no enhanced the uricase activity due to the accumulation of uric acid within the cell inhibited the uricase activity[10]. Ram *et al.*[10] reported that uric acid concentration higher than 0.3% did not enhance the uricase

production by *Xanthomonas fuscans* subsp *aurantifolii* due to a high concentration of uric acid inhibited the production of uricase. While Geweely and Nawar[28] reported that the highest extracellular and intracellular uricase production by *A. niger* were obtained at 0.1% of uric acid. The enhancement of uric acid concentration up to 0.2% had no affect on the uptake of uric acid in the cell due to the accumulation of urate within cell.

Lactobacillus plantarum Dad-13 is a kind of mesophilic lactic acid bacteria which grows well between 20 and 42°C while 30°C is the best temperature for growth of this strain[29]. Evaluation the effect of temperature on uricase production showed that uricase cannot be produced by *L. plantarum* Dad-13 when grown at 20°C due to highest glucose residue in the medium and lowest of intracellular of uric acid. Uricase activity increased with increasing temperature up to 37°C and then decreased at 42°C. Jagathy *et al.*[25] reported that optimum temperature for uricase activity by *A. niger* reached at 45°C while Geweely and Nawar[28] reported that optimum temperature for extracellular and intracellular uricase produced by *A. niger* reached at 27°C respectively. It is suggested that uricase production by some microorganisms were temperature dependent.

The lowest intracellular uric acid reached when *L. plantarum* Dad-13 grows at 20°C due to the lower temperature lead to decreasing of transportation. According to uricase activity, the result showed that intracellular uric acid at 37°C was less than at 30°C due to the higher uricase activity oxidized more uric acid into allantoin.

CONCLUSION

Lactobacillus plantarum Dad-13 produced uricase which remain active in the stomach and small intestine. Uricase is an inducible enzyme produced at the stationary phase of *L. plantarum* Dad-13 growth. The optimum uricase activity is obtained at temperature 37°C, on 0.15% of uric acid for 22 h incubation and can be used for hyperuricemia treatment.

SIGNIFICANCE STATEMENT

The development of probiotic lactic acid bacteria such as *L. plantarum* Dad-13 produces intracellular uricase with stability in gastrointestinal system is a promising potential therapy for hyperuricemia through oral route. This study will provide a scientific information for the treatment of hyperuricemia using lactic acid bacteria as functional food.

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