Selection of uric acid oxidizing-\textit{Lactobacillus plantarum} isolates based on their genetic determinant and uricase kinetics

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\textbf{ABSTRACT}

Hyperuricemia is a condition characterized by abnormally elevated levels of uric acid in the blood. It has been a leading morbidity disease. Microbial uricase can be used to oxidize uric acid into allantoin and hydrogen peroxide in the presence of oxygen and therefore has the potential to play an essential role in reducing uric acid in the people suffering from degenerative disease of hyperuricemia. The present study aims to select uric acid oxidizing-\textit{Lactobacillus plantarum} isolates based on their genetic determinant and uricase kinetics. A collection of \textit{Lactobacillus plantarum} isolates were grown on a selective differential medium followed by measuring their uricase activity spectrophotometrically. Specific primers for detection of uricase gene were designed. The uricase coding gene (uox) was then detected in all of the selected isolates by using a qPCR method employing the designed specific primers. The uricase kinetics was determined by the Lineweaver-Burk method. Results showed that all isolates had uricase activity and 4 potential isolates were selected based on their superior uricase activity. The uox gene was detected in all of the selected isolates. The kinetics analysis, however, revealed that only the \textit{L. plantarum} K-Mar-A2 show strongest substrate affinity and was considered a potential candidate to be developed as a source of therapeutic agent for hyperuricemia.

\textbf{INTRODUCTION}

Hyperuricemia is a condition characterized by abnormally elevated levels of serum uric acid (Benn \textit{et al.}, 2018). Hyperuricemia has been a leading morbidity disease. It has been shown to be a risk factor for gout (Fang \textit{et al.}, 2020; Zhang \textit{et al.}, 2020) the most common form of inflammatory arthritis, arises from the subsequent deposition of urate crystals when concentrations become saturated (Benn \textit{et al.}, 2018). Uric acid (2,6,3 trihydroxypurine) is the insoluble end product of exogenous and endogenous purine catabolism. The relationship between diet and serum uric acid seems to be more complex than simple purine intake, as beer and high fructose soft drinks have been shown to influence serum uric acid levels irrespective of their purine content (Benn \textit{et al.}, 2018).

The biosynthesis of uric acid is catalyzed by the enzyme xanthine oxidase also known as xanthine oxidoreductase. The enzyme is widely distributed throughout various organs including the liver, gut, lung, kidney, heart, and brain as well as the plasma and plays roles in two stages of uric acid production:
conversion of hypoxanthine to xanthine and subsequently xanthine to uric acid (Benn et al., 2018).

In most mammalian species uric acid is further metabolized by the enzyme uricase to the more soluble metabolite which is subsequently excreted in the urine (Benn et al., 2018). Human and certain primates, however, lost their uric acid oxidizing enzyme, the uricase, consequently uric acid is the final product of purine catabolism and can not be metabolized to the soluble allantoin, urea, or ammonia for excretion from the body (Kratzer et al., 2014; Iswantini et al., 2014; Tan et al., 2016).

Interestingly, uricase is also encoded by a number of microorganisms and the enzyme has been found in microbes such as Pseudomonas aeruginosa, Bacillus fastidiosus, Bacillus subtilis subsp. subtilisstr. 168 and Bacillus subtilis subsp. spizizenii TU-B-10 (Abdel-Fattah et al., 2005; Pfrimer et al., 2010; Tan et al., 2012).

Notably, an isolate of probiotic bacteria of Lactobacillus plantarum has been reported to show an uric acid oxidizing activity (Iswantini et al., 2014). L. plantarum is commonly found in plant-derived foods such as fruits, pickles, wine, and bean sauces (Li et al., 2014). It is essential then to explore, select and characterize its uricase in term of both genetic and kinetic properties which is important for developing a potentially probiotic for hyperuricemia.

MATERIALS AND METHODS

Forty six L. plantarum isolates were collected from the fruit of mangosteen, eggplant, mango, and passion fruit. The culture medium was a selective differential GYP (1% glucose 1% yeast 0.5% peptone b/v) liquid and agar medium supplemented with 0.3% b/v uric acid. Uricase activity in the supernatant fraction was measured spectrophotometrically.

Culture and selection of L. plantarum

All L. plantarum isolates were grown on GYP agar medium with CaCO₃ for 24 hour at incubation 37°C. The uric acid (0.3% b/v) was supplemented to GYP broth in the selection process. The amount of uric acid oxidized was measured spectrometrically at λ 595 nm and reported as relative the control medium without bacterial treatment.

Primer Design for the uox gene

Both forward and reverse primers of uox gene were derived from the uox sequence alignment of Bacillus subtilis subsp. subtilis str. 168, Bacillus subtilis subsp. spizizenii TU-B-10, Pseudomonas putida KT2440, Pseudomonas aeruginosa PA01, Amycolatopsis vancoressymicina strain NRRL B-24208, and Streptomyces venezuelae ATCC 10712. The Primer-BLAST and Clustal Omega program were used in the primer design. The primers were synthesized by IDT (Integrated DNA Technologies) of Singapore.

Detection of uox gene

The bacterial DNA was isolated through a heatshock technique of at 95°C for 15 minutes, then cooled down to -20°C for 5 minutes. The q-PCR reaction of 20 µL was performed in a Bio-Rad CFX of 40 cycles: 3 min pre-denaturation at 95°C, 10 sec denaturation at 95°C, and 30 sec annealing at 55°C.

Uricase Assay

The selected L. plantarum isolates were grown on Glucose Yeast Peptone (GYP) broth for 24 hour at incubation 37°C. Crude extract of uricase were harvested by centrifugation (10000 rpm, 14 min). The crude extract of uricase activity was measured spectrophotometrically at λ 293 nm.

Characterization of the selected L. plantarum isolates

The selected L. plantarum isolates were subjected to measurement of activity of crude extract uricase at pH and temperature range: pH 6; 7; and 8, and temperature 25; 30; and 37°C. Michaelis-Menten constant (Kₘ) and maximum velocity (Vₘₐₓ) of crude extract uricase based on various substrate 2-7 mg/dL at the best pH and temperature, then plotted into a Lineweaver-Burk curve.

RESULTS AND DISCUSSION

Uric acid oxidizing activity of L. plantarum

In this study, we report uric acid oxidizing activity in strains of L. plantarum, isolated from tropical fruits in Indonesia. The fruit’s sweet and acidic condition would support the presence of lactic acid bacteria. The mannose-rich fruit mesocarp would be the best fruit part to find the mannose specific adhesin (MSA)-producing L. plantarum. The MSA plays important roles in the probiotic interaction with host intestinal tract, immunologic responses and health related benefits (Gross et al., 2010; Pretzer et al., 2005; Nieuwboer et al., 2016).

All of the 46 L. plantarum isolates were be able to oxidize the uric acid down to 3.6 to 82.1 percent. The top ten uric acid oxidizing isolates where presented in Figure 1. The four best isolates, K-Mar-A2, Mar-A18, Mgs-Bst-3, and Mgs-Psmb-3 were selected and then subjected to uox gene identification and uricase enzyme kinetics analysis (Figure 1).

Detection of uox gene

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The four selected isolates were tested for the presence of the uox gene. The best designed primers were a forward primer: TGATCGCTTCCGCTTTCCCTT and a reverse primer: TCCGGGATTTCCTTCCTCC. The uox gene was detected in all of the four selected isolates and their qPCR profiles were presented in Figure 2. The K-Mar-A2 isolate showed better qPCR signal than the other isolates. Specific primers were created to identify uox gene by qPCR. The best designed primers successfully amplified the uox gene in the four isolates, and the K-Mar-A2 isolate showed the best qPCR signal compared with the other selected isolates.

Our data confirmed the presence of uox gene in every isolate tested which encodes the uricase enzyme with detected activity in each isolate. The detected uricase genes of the four selected isolates can potentially be used to produce recombinant uricase by cloning and expressing the genes in host cells. Production of recombinant microbial uricase has been reported (Cheng et al., 2012).

Recombinant *Aspergillus flavus* uricase (Rasburicase) has been used for the treatment of gout and hyperuricemia occurring in tumor lysis syndrome. Compared to allopurinol, a competitive inhibitor of xanthine oxidase, a common agent for blocking the conversion of hypoxanthine and xanthine to uric acid, thereby, reduces uric acid production,
the use of Rasburicase has advantages, in that, it reduces pre-existing hyperuricemia and does not cause accumulation of xanthine or hypoxanthine which occurs after allopurinol uptake (Imani and Shahmohamadnejad, 2017).

**Kinetics of L. plantarum uricase**

Our results show that the uricase works better at pH 6 and 25°C (Figure 3). As expected from genetic analysis, the K-Mar-A2 isolate showed a better catalytic rate and substrate affinity as revealed by the Lineweaver-Burk curves (Figure 4). The urate oxidase expressed by K-Mar-A2 isolate exhibited uric acid oxidase activity, with crude enzyme activity 0.4028 U/mL with $K_M$ and $V_{max}$ were 0.0137 mg/mL and 0.1311 μmol mL$^{-1}$ min$^{-1}$.

The Lactobacillus plantarum uricase of our study showed different optimum temperature and pH from microbial uricase previously reported. Characterization of bacterial uricase from Sphingobacterium thalpophilum (VITPCB5) showed that the optimum temperature of the enzyme activity was between 25 and 45°C, and the optimum pH was 8.0 (Ravichandran et al., 2015). Studies of purified uricase of Pseudomonas aeruginosa showed optimum temperature of 30°C and optimum pH of 9.0 (Saeed et al., 2004). The kinetic data presented in the present study were obtained from crude enzymes. Further experiments are required to determine the kinetics of pure Lactobacillus plantarum uricase.
CONCLUSIONS

This study revealed the uricase might be common in *L. plantarum*. The selected isolate might be developed as a source of therapeutic agent for hyperuricemia. More specifically, results showed that the *L. plantarum* K-Mar-A2 isolate is the best candidate for uric acid oxidizing probiotic bacterium.

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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