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To cite this article: A Frediansyah *et al* 2019 *IOP Conf. Ser.: Earth Environ. Sci.* **251** 012045

IOP Conf. Series: Earth and Environmental Science **251** (2019) 012045 doi:10.1088/1755-1315/251/1/012045

Lactobacillus pentosus isolated from *Muntingia calabura* shows inhibition activity toward alpha-glucosidase and alpha-amylase in intra and extracellular level

A Frediansyah[1], Suryani, R Nurhayati[1], Miftakhussolikhah[1], J Sholihah

[1]Research Unit for Natural Product Technology, Indonesian Institute of Sciences Jl. Yogya-Wonosari km 31.5 Gading, Playen, Gunungkidul, Yogyakarta, Indonesia

Email: andri.frediansyah89@gmail.com

Abstract. *Lactobacillus pentosus* is one of the most prevalence lactic acid bacterium which available in plant-based fermentation. Here, we reported that the cell extract and cell-free supernatant of *L. pentosus* strain TL 2.7, TL 5.8 and TL 7.8 isolated from Kersen (*Muntingiacalabura* L.) showed potential anti-diabetic properties by inhibition of alpha-glucosidase and alpha-amylase activity *in vitro*. All isolated also enhanced the antioxidant capacity through DPPH *in vitro* test. This initial study will be beneficial for screening and application of functional food with anti-diabetic properties.

Keywords. *Lactobacillus pentosus*, inhibitor alpha-glucosidase, *Muntingia calabura,* Kersen, alpha amylase

1. Introduction

Diabetes mellitus type 2 is one of the significant contributions to the world's mortality rate. The report by the International Diabetes Federation in 2015 showed that about 415 million adults have diabetes[1]. Based on its prediction, the number of diabetes cases will increase 1.5 times higher by 2040. People with diabetes have poor insulin performance; this will lead to the elevation of blood glucose after food consumption. This stage is called postprandial hyperglycemia that results in numerous complication including diabetic foot ulcer, nephropathy, retinopathy, and neuropathy [2]. During the digestion in gastrointestinal (GI) tract, the 1,4 alpha glycosidic bond from the polysaccharide is cleaved by alpha-amylase to produce disaccharide or oligosaccharide, which later then hydrolyzed to monomers such as glucose in the presence of alpha-glucosidase. The resulting product then absorbed through the small intestine to the portal vein of the liver [3, 4]. Therefore, the presence of these inhibitors can inhibit or prevent the increasing level of blood glucose in our body.

Acarbose, voglibose, and miglitol are common synthetically drug for people with diabetes who are easily found in the market [5]. However, miglitol and voglibose remain not effective on the inhibition toward alpha-amylase. The use of synthetically material as a drug is often connected to the number of GI side effects. Moreover, about the three-quarters population with diabetes are living in non-developed countries and the high price of drugs often being one of the major problems [1]. Recently, bioactive compound is demanding to a massive group of people due to the high number of the positive outcome. This material also gives fewer side effects on the human body. Up to now, scientists have put their effort to find a way out of the risk of diabetes mellitus type 2 by finding alpha-amylase and alpha-glucosidase inhibitor from many sources such as fungi [6, 7], plants [8], marine creatures [9], and bacteria [10]. However, the research related bacteria with its anti-diabetic activity are still limited.

While bacteria especially lactic acid bacteria can be employed as a part of a functional food with anti-diabetic properties.

Muntingia calabura L, known as Kersen, is easily found in the land of Indonesia. The leaf reputed to possess anti-diabetic effect by *in vivo* study using alloxan-induced diabetic rats (*Rattus norvegicus*) [11]. Flavanoid content on the Kersen's leaf has a good activity toward alpha-glucosidase inhibition [12]. On the fruit part, *M. calabura* contain diabetic substance such as beta-carotene, riboflavin, niacin, thiamine, and ascorbic acid. Its anti-diabetic properties have been demonstrated by *in vivo* experiment using rats induced by streptozotocin [13]. Therefore, this study was conducted to isolate and characterize some potential bacteria with anti-diabetic activity from the fruit part of *M. calabura*.

2. Material and Methods

2.1. Materials

Kersen fruits (*M. calabura*) was obtained from the garden of the Research Unit for Natural Product Technology - Indonesian Institute of Sciences (BPTBA-LIPI), Yogyakarta, Indonesia in 2016.

2.2. Isolation of bacteria

The fruit sample was cleaned using tap water and sterilized by consecutive washes in 75% methanol for about 1 minute and rinsed with sterilized distilled water three times. About 20 grams of samples were taken and fermented with MRSB medium 100 ml at 37 °C for 48 hours. The isolation procedure was done using MRSA medium mixed with calcium carbonate by pour plate method. Colonies with halo zone were selected and purified on the MRSA medium by streak plate method. Several colonies with various shapes and size were picked and purified using MRSA medium by streak plate method and re-purified in the same medium for three times. The incubation was employed at 37 °C for 48 hours.

2.3. Characterization and molecular identification using 16S RNA

The single colony was then characterized based on physical and chemical properties including the shape of the cell, gram staining, motility, catalase test, and endospore test. For molecular identification, the bacterium was extracted using a modification method by Giraffa et al. 2000 [14]. The extracted DNA was used as a template for PCR amplification. DNA was then used as a template for PCR to amply the 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') from Promega. The result of sequencing was then compared with the National Center for Biotechnology Information (NCBI) GenBank database. The sequence was aligned with CLUSTALW parameter which includes pairwise and multiple alignments. Gap opening penalty and gap extension penalty were 2 and 0.8, respectively. A final phylogenetic tree was constructed using MEGA software (version 7.0.0).

2.4. Preparation of cell extract and cell-free supernatant

The strains of *L. pentosus* were inoculated in 5 ml MRSB medium, then incubated for 24 h at 37 °C. The medium contained cell then centrifuged at 4 °C, 10000 g, for 15 min. The biomass cell will occur at the bottom of the tube and resulting cell-free supernatant. The biomass cell was then extracted by ultra-sonication with methanol and subsequently filtrated with 0.22 μm membrane filter (Millipore). The supernatant and extracted cell were used for the activity assay.

2.5. Measurement of pH

The pH of bacterial activities during incubation was measured at 24, 48, 72 and 96 hours. The test was calculated with three independent replications.

2.6. Antioxidant scavenging activity

The 1.1-diphenyl-2-picrylhydrazyl called as DPPH; scavenging assay was performed using the 96-well plate as the previous report in Frediansyah et al. 2017 [15]. Briefly, a filtered cell-free supernatant was mixed with methanol, and the absorbance of the pre-plate reading was recorded at 517 nm. DPPH solution in methanol was then added to the well. The changing color from purple to yellow visualized the scavenging efficiency of the cell-free supernatant. After the incubation period of 30 min at room

temperature in the darkness, the change of the absorbance was monitored at 517 nm in the Multiskan® Go micro-plate spectrophotometer (Thermo Scientific, Vantaa, Finland). The reduction of absorbance in the reaction showed a higher free radical-scavenging activity. The scavenging activity against DPPH was calculated using the equation (a):

$$\text{Scavenging rate (\%)} = [1 - (\text{AbsB} - \text{AbsA})] \times 100\ \% \qquad \text{(a)}$$

Where AbsA was the absorbance of control and AbsB was the absorbance in the presence of cell-free supernatant.

2.7. Alpha-glucosidase inhibition activity

The inhibition activity toward alpha-glucosidase from cell extract and cell-free supernatant were analyzed according to previous reports [15]. In brief, the mixture contained the sample, alpha-glucosidase, and sodium phosphate buffer. The mixture was then incubated at 37 °C for 15 min. After pre-incubation, the enzymatic reaction was initiated by implemented *p*-nitrophenyl-α-D-glucopyranoside solution in the phosphate buffer. The resulting reaction mixture was incubated for 20 min at 37 °C. The absorbance was subsequently measured at 405 nm in the Multiscan® Go microplate spectrophotometer (Thermo Scientific, Vantaa, Finland). Percent inhibition was calculated by a formula (b):

$$\%\ \text{inhibitor} = [(\text{Abs1} - \text{Abs2})/\ \text{Abs1}] \times 100\ \% \qquad \text{(b)}$$

Where Abs1 is the absorbance of the control and Abs2 is absorbance in the presence of the sample.

2.8. Alpha-amylase inhibition activity

The inhibition activity toward alpha-amylase from cell extract and cell-free supernatant were measured according to Telagari and Hullatti, 2015 [16]. The test system was performed in a 96-well plate. Briefly, the mixture contained the sample, alpha-amylase, and sodium phosphate buffer. The mixture was then incubated at 37 °C for 20 min. After pre-incubation, the soluble starch was then added to a sodium phosphate buffer as a substrate. Then, the aliquot was incubated further at 37 °C for 30 min, followed by adding 3.5-di-nitrosalicylic acid (DNS) solution and heated at 100 °C for 10 min. The absorbance was subsequently measured at 540 nm on Multiskan® Go microplate spectrophotometer (Thermo Scientific, Vantaa, Finland). The percentage of α-amylase inhibition was calculated by the same formula in 2.7.

3. Result and Discussion

3.1. Strain characterization and taxonomical position

Three strains of *L. pentosus* from *M. calabura* fruit have been isolated and characterized based on physical and chemical properties, and also 16S rRNA. Based on physical and chemical properties as shown in Table 1, these three strains of *L. pentosus* belongs to lactic acid bacteria. This bacterial clade gives the positive reaction to gram staining, negatively react to catalase, non-motile and not forming spore [17]. Based on information from 16S rRNA gene sequencing, TL 1.7, TL 5.8 and TL 7.8 have 100%, 99% and 99 % similarity to *L. pentosus* strain 124-2, respectively.

Table 1. Physical and chemical properties of three strains of *L. pentosus* isolated from *M. calabura*

Strains	Cell form	Gram staining	Catalase test	Motility	Endospore	Identity
TL 1.7	Rod	+	-	-	-	Lactic acid bacteria
TL 5.8	Rod	+	-	-	-	Lactic acid bacteria
TL 7.8	Rod	+	-	-	-	Lactic acid bacteria

The taxonomical tree, as shown in Figure 1, showing the relative position of *L. pentosus* strain TL 2.7, TL 5.8 and TL 7.8 as inferred by the neighbor-joining method with the 16S rRNA gene sequence

from *L. pentosus* strain 124-2 as obtained from National Center for Biotechnology Information (NCBI) as the in-group sequence. The optimal tree has branch length 0.2269 and bootstrap values for a total 1,000 replicates. The evolutionary distances were computed using the Kimura 2-parameter method and in the units of the number of base substitutions per site.

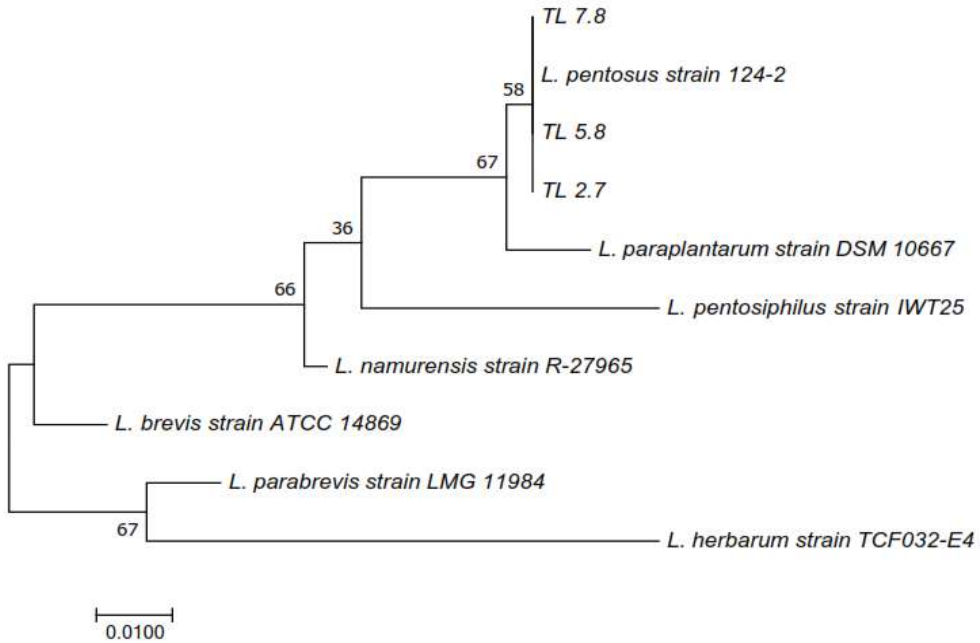

Figure 1. Phylogenetic relationship of *L. pentosus* TL 2.7, TL 5.8 and TL 7.8 based on 16S rRNA gene sequence.

3.2. Antioxidant activity and pH

The antioxidant activity from the supernatant of MRSB medium containing *L. pentosus* was investigated using DPPH free radical scavenging activity assay as shown in Figure 1. The reducing capacity of antioxidant was measured spectrophotometrically based on the color change from dark violet to yellow. The presence of an antioxidant in the bacterial medium after incubation can decrease the free radical by electron donor or hydrogen atom transfer.

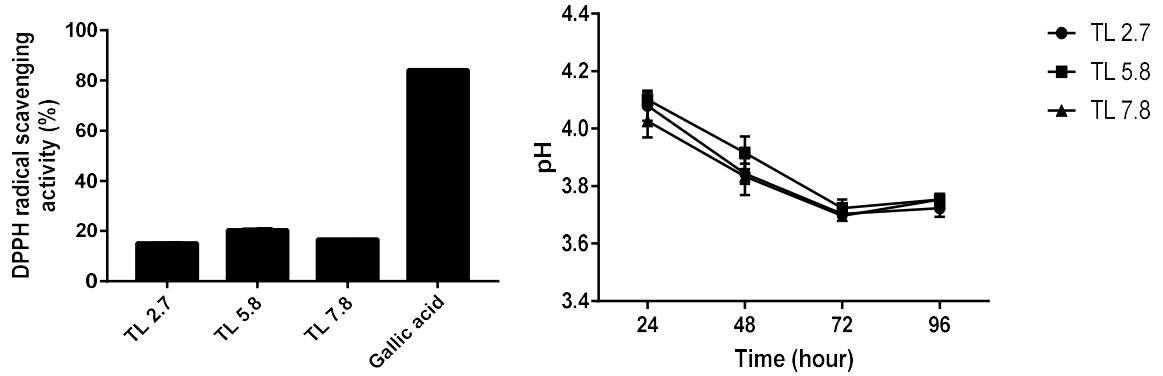

Figure 2. Antioxidant level of *L. pentosus* in MRSB using DPPH radical scavenging activity assay (left) and pH level of MRSB medium containing *L. pentosus* during 96h incubation (right)**.**

All strains of *L. pentosus* showed scavenging activity (Figure 1). However, these activities only show about 4.12 to 5.53 times lower compare with Gallic acid as a positive control. Each result was

significantly different at $p \leq 0.01$ using ANOVA followed by Tukey's HSD test. The value for TL 2.7, TL 5.8 and TL 7.8 are 15.21%, 20.44% and 16.68%, respectively. This finding revealed that *L. pentosus* is valuable in enhancing the antioxidant level in MRSB medium. Nevertheless, this result showed lower scavenging activity compared with *L. plantarum* in the medium containing black grape juice [16]. Additionally, the pH level decreased during incubation. Hachemet al 2005 [18] stated that the pH could enhance the activity of an enzyme, while it also could improve the bioavailability of compounds. The elevation of antioxidant level due to the presence of bacteria in this experiment is in agreement with many reports.

3.3. Inhibition of alpha-glucosidase and alpha amylase

Postprandial hyperglycemia is the valuable target as first-line therapy for people with diabetes. One of the ways to reduce that step is to retard the monosaccharide absorption by inhibiting alpha-glucosidase and alpha-amylase. Many studies have been conducted to inhibit these enzymes by the natural compound from a plant, including seeds, roots or fruits. Limited research has been employed on the anti-diabetic potential of lactic acid bacteria.

Cell-free supernatant and cell extract of each strain of *L. pentosus* showed good inhibition against two enzymes related to diabetes (Figure 2). Among them, cell extract showed higher restraint to alpha-glucosidase compare to the cell-free supernatant group. However, this finding is not applicable to the inhibition of alpha-amylase. Among three strains of *L. pentosus,* TL 2.7 showed the highest inhibition activity again α-glucosidase and α-amylase to both from its cell-free supernatant and cell extract. This result is not far different with these activities from lactic acid bacteria isolated from *Canna edulis* and *Xanthosoma sagittifolium* [19] and *Ganoderma lucidum* [20]. *L. pentosus* has a good potential as inhibitor alpha-amylase which is opposite to *Paenibacillus spp*. [21],*L. plantarum* [22, 23], and *L. acidophilus* [24] which are good as amylase producer. However, acarbose, a positive control in this experiment, showed higher inhibitor activity with the value of 88.22% compared with those three strains, but only restricted to inhibition of alpha-glucosidase. In detail, the inhibition of alpha-glucosidase by cell extract of TL 2.7, TL 5.8 and TL 7.8 are 1.04, 1.070 and 1.27 times lower, respectively. From the cell-free supernatant with similar order showed 1.26, 1.29 and 1.33, respectively. While for α-amylase inhibitor showed the opposite trend, all those results both from the cell-free supernatant and cell extract gave higher inhibition compare to Acarbose 1 mg.

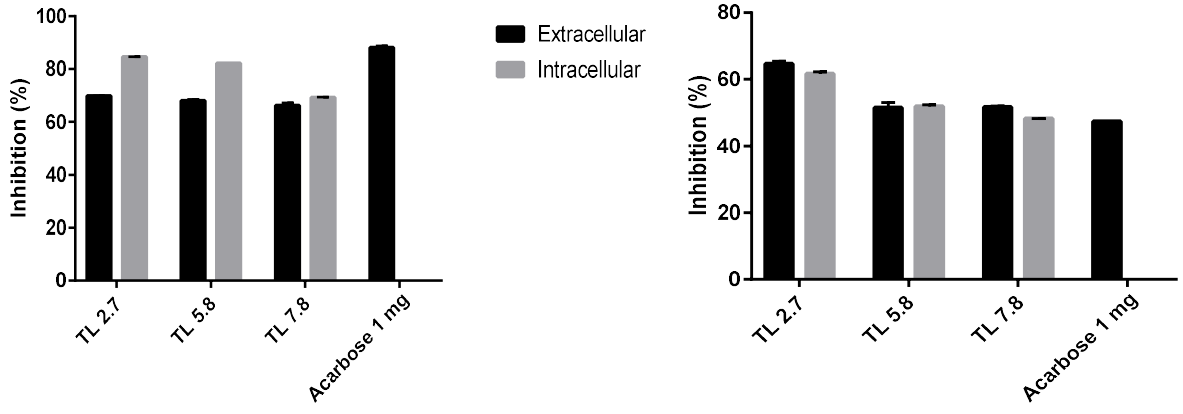

Figure 3. Alpha-glucosidase and alpha-amylase inhibition activities of *L. pentosus* strain TL 2.7, TL 5.8 and TL. 78. The left graph (A) represented inhibition α- glucosidase from an extract of the cell and cell-free supernatant from various strains of *L. pentosus*.

4. Conclusion

The current *in vitro* study showed that *L. pentosus* (TL 2.7, TL 5.8 and TL 7.8) has good potential as anti-diabetic properties by inhibition of alpha-glucosidase and alpha-amylase activity. This bacteria is also valuable to enhance antioxidant capacity. This initial study will be beneficial for screening and application of functional food with anti-diabetic properties.

5. Acknowledgment

We gratefully acknowledge the financial support of internal sources funding of DIPA LIPI. We also thank lab members in Food and Microbiology Laboratory for their valuable contributions during preparation.

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