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Isolation and identification of endophytic bacteria from Salak Pondoh (*Salacca edulis*) fruit as α-glycosidase inhibitor producer

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Abstract

Alpha-glycosidase inhibitors can delay the hydrolysis of oligosaccharide and disaccharide into glucose, which can prevent or treat hyperglycemia in diabetes mellitus. The rind and flesh of Salak Pondoh fruit are known to produce α-glycosidase inhibitor compound. Endophytic bacteria that live in plant tissues potentially produce compounds such as in host plants. Exploration of endophytic bacteria from the rind and flesh of Salak Pondoh is one of the efforts to obtain isolates of bacteria producing α-glycosidase inhibitors. The objective of this study is to isolate and identify endophytic bacteria producing inhibitor α-glycosidase from rind and flesh of Salak Pondoh, and to know the activity of its α-glycosidase inhibitor. Isolation of endophytic bacteria was done by inoculating surface-sterilized fruit samples on Nutrient Agar (NA) medium. The inhibitory activity towards α- glycosidase analysis was performed using the spectrophotometric method (λ = 415 nm), with p-nitro phenyl α-D-glucopyranoside as the substrate. Identification of the bacteria was performed based on the 16S rRNA gene sequence. The sequencing was done at 1st Base Singapore and the obtained sequences were identified using the BLAST Nucleotide device on the NCBI website. In total, 6 endophytic bacterial isolates were obtained. The inhibitory activity ranged from 6.14-62.95% and the highest was generated by Kt-E isolates. The isolated bacteria were Dt- A and Dt-B that represent genus Xanthomonas, Kt-E from genus Paenibacillus, Kt-I from genus Bacillus, Dm-A1 and Dm-A2 from family Enterobacteriaceae. The results confirm the potential of the endophyte bacteria of Salak Pondoh to be an alternative source of hyperglycemia medication.

How to Cite

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INTRODUCTION

α-glycosidase is an enzyme located along the microvillus of human›s small intestines. It plays a role in the hydrolysis of α-1.4-glycosidic bond in oligosaccharides or disaccharides into glucose which is then absorbed by blood vessels and enter the bloodstream (Sulistiyani et al., 2016). In normal condition, this postprandial blood glucose will be circulated by insulin to the target cells. However, on those who suffer from diabetes mellitus, the postprandial glucose accumulates in the blood due to insulin resistance or insulin production abnormalities. It causes hyperglycemia in diabetes mellitus. Hyperglycemia is a condition when the blood glucose levels are higher than normal. Chronic hyperglycemia can lead to complication of disease in people with diabetes mellitus, such as coronary heart disease, stroke, renal failure, and hypertension (Harris, 2000).

Inhibition of α-glycosidase enzyme activity is one of the preventions and treatments against hyperglycemia in diabetes mellitus. The α-glycosidase inhibitory measure could delay the hydrolysis process of oligosaccharides or disaccharides and prevent hyperglycemia in diabetes mellitus patient (Sulistiyani et al., 2016; Shinde et al., 2008). Tadera et al. (2006) and Xu (2010) suggested that flavonoids in the form of glycosides could inhibit α-glycosidase by binding to the active side of the α-glycosidase enzyme. A commercialized α-glycosidase inhibitor for diabetes treatment such as acarbose is generated by Actinoplanes sp. (Li et al., 2012).

An α-glycosidase inhibitor can be extracted from fresh rind and flesh of Salak Pondoh. The inhibitory activity of rind and flesh extracts of Salak Pondoh ranged from 0.716695-30.10119%. However, according to Priyanto et al. (2014), the production of secondary metabolite compounds by extracting from plants takes a long time due to the relatively long plant life cycle. Alternatively, utilization of endophytic bacteria living in the plant tissue could be a potential new source. Tan and Zhou (2001), stated that endophytic bacteria is expected to produce secondary metabolite compounds similar to its host plants. Priyanto et al. (2014) also suggested that the time required for the production of secondary metabolite compounds from endophytic bacteria is shorter because the bacterial life cycle is relatively faster compared to plants. Based on these findings, endophytic bacteria in the rind and flesh of Salak Pondoh might also be able to produce secondary metabolite compounds that inhibit α-glycosidase.

It is necessary to conduct research concerning the potential of endophytic bacteria from Salak Pondoh fruit for α-glycosidase inhibitor production as a potential alternative for hyperglycemia medication for people with diabetes mellitus. The objective of this study is to isolate and identify endophytic bacteria producing α-glycosidase inhibitors from rind and flesh of Salak Pondoh fruit, as well as to know the activity of their α-glycosidase inhibitor.

METHODS

Plant collection and sample preparation

Samples of young (± 3 months after pollination) and mature fruit (± 6 months after pollination) of Salak Pondoh were collected from Agromulyo Village, Cangkringan subdistrict, Sleman, Yogyakarta. The flesh was separated from the thin rind. The rind and the flesh then were surface-sterilized by series of soaking in 70% ethanol for 3 minutes, in 3% commercial bleaching solution for 5 minutes and lastly in 70% ethanol for 30 seconds. Sterilized samples then were rinsed three times using distilled water. The success of surface sterilization was checked by inoculating 100 mL of distilled water from the final rinsing on Nutrient Agar (NA) medium at 25°C for 7 x 24 hours (Sulistiyani and Lisdiyanti, 2016).

Isolation and purification of bacteria

Bacterial isolation was done using the plant piece (sample pieces) method. The flesh and the rind were cut into ± 1 cm2 size and then were placed on NA medium separately. Incubation was carried out at 25°C for 7x24 hours. Grown bacteria were purified using the quadrant method on NA media until a single colony was obtained. The pure bacterial isolates were stored in a sloping NA medium (Sulistiyani and Lisdiyanti, 2016 and Pujiyanto et al., 2015).

Morphological characterization of endophytic bacteria

Macroscopic characterization

Colonies of endophytic bacteria were characterized based on their colour, shape, edge shape, and elevation or angle of protrusion of colony growth on NA medium.

Microscopis characterization

The microscopic characteristics of bacterial isolates were distinguished by using gram staining method. Before the staining procedure, a heat-fixed smear was prepared by smearing the bacteria on a glass slide that previously washed

with 70% alcohol and added with a drop of physiological saline (NaCl 0.85%) and then heated over Bunsen burner flame with the smear-side up. Staining was done by adding 2-3 drops of primary stain (crystal violet) on the smear for 1 minute and was washed with flowing distilled water. Second reagent, Gram's iodine solution was added and let to sit for 1 minute before being washed with distilled water. Next was adding decolorizing agent, acetone:alcohol 96% (1:1) for 10-20 second. After being washed with distilled water, counterstain reagent (safranin) was added last for about 20-30 second and being washed again with water. Bacteria were observed with a 400x magnification microscope. Purple colour indicates gram-positive bacteria whereas red colour indicates gram-negative bacteria (Pujiyanto et al., 2015).

Test for inhibition of enzyme a- glucosidase

Pure culture of endophytic bacteria was cultured on YPS (Yeast Peptone Starch soluble) media containing 0.1% starch powder, 0.5% peptone, and 0.15% yeast extract at pH 7, and agitated at 90 rpm for 2x24 hours at a temperature of 28°C. Cultured bacteria were subsequently centrifuged at 3500 rpm for 15 min to separate the metabolite extracts with the bacteria. The metabolite extracts obtained in the supernatant was further tested for its inhibition of the enzyme α-glycosidase (Pujiyanto et al., 2015).

Enzyme solution 1 unit/ml was made by dissolving 2.695 mg of α-glucosidase (Sigma-Aldrich® G5003) on 50 ml of phosphate buffer pH 7 (Immortals, 2012). A reaction mixture containing 2 µl metabolite extract in the supernatant, 48 µl phosphate buffers (100 mM, pH 7) and 25 µl α-glucosidase enzyme (0.25 units/ml) was incubated at 37°C for 5 minutes. After incubation, 25 µl of substrate p-nitrophenyl α-D-glucopyranoside (Sigma Aldrich®, N1377) (20 mM) was added to the mixture. Further incubation for 15 min at 37°C was carried out and then 100 µl sodium carbonate/Na2CO3 200 mM was added to stop the reaction. The absorbance was read using a microplate reader at a wavelength of 415 nm (Pujiyanto et al., 2015). Acarbose solution of 1% was prepared as a comparison with enzymatic reaction of all extracts (positive control) by dissolving powder tablets Glubose® into phosphate buffer pH 7 (1: 100) (Sulistiyani et al., 2016). The complete enzymatic reaction design for one sample with a total volume of 200 µl can be seen in Table 1.

Table 1. Reactions test design of enzymatic inhibitory activity on α-Glucosidase (modified from Pujiyanto et al., 2015).

	Mixture			
	A_0 (µl)	A_1 (µl)	AI_0 (µl)	AI_1 (µl)
Supernatant	-	-	2	2
Media YPS	2	2	-	-
Buffer pH 7	48	48	48	48
Enzim	-	25	-	25
Incubated at 37°C for 5 minutes				
Buffer pH 7	25	-	25	-
Substrate	25	25	25	25
Incubated at 37°C for 15 minutes				
Na_2CO_3	100	100	100	100

The inhibitory activity of the extracts and Acarbose on α-glycosidase enzyme can be calculated using the following formula: % inhibition = (A1 absorbance - A0 absorbance) - (AI1 absorbance-AI0 absorbance) / (A1 absorbance - A0 absorbance)*100% (Puspitayanti, 2017).

Amplification of the 16S rRNA encoding gene

The bacterial isolates producing the α-glycosidase inhibitor were cultured on NB media and agitated at 50 rpm for 18 hours at 28°C. The DNA extraction was performed using Presto TM Mini gDNA Bacteria Kit. The genome of the extracted DNA was determined for its purity and concentration using a bio-photometer. The DNA of the genome was amplified, DNA was determined to be pure if the absorption of A260/280 ranges from 1.8-2 (Kaeppler-Hanno et al., 2015).

The genes encoding 16S rRNAendophytic bacterial was amplified using Polymerase Chain Reaction (PCR) by mixing 25µl MyTaq TM Red Mix polymerase, 1 µl 63 forward primer (63f) and 1 µl 1387 reverse primer (1387r) with concentrations of each 10 pmol, 100 ng DNA template, and ddH2O was added up to a volume of 50 µl. Pre-denaturation was carried out at 95°C for 1 minute. The PCR cycle was 30 cycles. One PCR cycle consisted of denaturation at 95°C for 15 seconds, annealing at 55-58°C for 15 seconds, and elongation at 72°C for 10 seconds, then the cycle was stopped and stored at 4°C (Marchesi et al., 1998). The amplicon of 16S rRNA gene was electrophoresed (100 mA, 85V, for 30 min), then was sequenced at 1st Base Singapore. Sequences of 16S rRNA genes were then analyzed using BLAST Nucleotide device on the NCBI website to identify the bacteria.

The identification of endophytic bacteria producing α- glycosidase inhibitor was based on the similarities between isolate's gene sequence with the GenBank. According to Drancourt et al. (2000) and Sperling et al. (2017), similarities more than 99% can be considered as the same species. If the similarities ranging from 97-99%, it's most likely the isolates share the same genus but different in species level. While, when the similarities are between 90-97%, then it only represents the same family and potentially is new species.

RESULTS AND DISCUSSION

Endophytic bacteria isolate producing α-glycosidase inhibitors

Endophytic bacteria from Salak Pondoh with the potential ability to produce a-glucosidase inhibitor were successfully isolated. Six isolates of endophytic bacteria were Dt-A, Dt-B, Kt-E, Kt-I, Dm-A1, and Dm-A2 (Fig. 1 and Table 2). All isolates have diverse colony morphological characteristics. Isolates Dt-A and Dt-B colony have similar morphological characteristics, both colonies are yellow in colour, glossy, slimy and categorized as gram-negative bacteria. However, their cell shape is different which are bacillus and coccus respectively.

Kt-E and Kt-I isolates are white-coloured, gram-positive, and bacillus-shaped cells. Specifically, Kt-E colony has elongated growth pattern on solid media, root-like form, rooted, crateriform and split colonies. While for the Kt-I isolate, the edges of the colony are irregularly shaped with a flat elevation. Dm-A1 and Dm-A2 isolates are gram-negative bacteria in the form of coccus. Characteristics of Dm-A1 and Dm-A2 colonies are having irregular shapes and wavy edges. However, it is different in colony colour, each of which is white and grey and yellowish white and somewhat slimy.

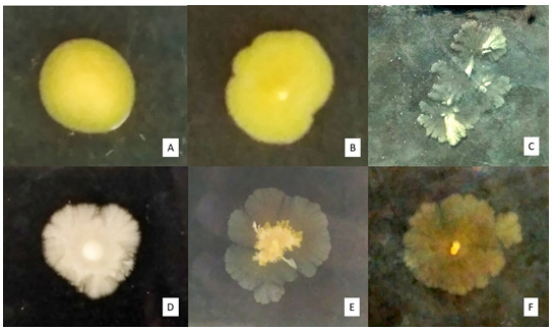

Figure 2. Isolate of endophytic bacteria Dt-A (A), Dt-B (B), Kt-E (C), Kt-I (D), Dm-A1 (E), and Dm-A2 (F) from rind and flesh of Salak Pondoh.Isolat Kt-E dan Kt-I mempunyai karakteristik morfologi koloni berwarna putih, gram positif, dan berbentuk basil.

The activity of α-glycosidase inhibitor

The inhibitory activity of α-glycosidase is indicated by a decrease in the activity of α-glycosidase in catalyzing the hydrolysis of p-nitrophenyla D-glucopyranoside into p-nitrophenol (yellow) and glucose after addition of bacterial culture supernatant containing α-glycosidase inhibitor. The six isolates of endophytic bacteria from the rind and the flesh of Salak Pondoh were able to produce α-glycosidase inhibitor, which is thought to be flavonoids glycosides similar to α-glycosidase inhibitor produced by the rind and the flesh of Salak Pondoh. The inhibitory activity ranged from 6.14 -62.95% and the highest was generated by Kt-E isolates (Table 3).

α -glycosidase inhibitor activity of six isolates of endophytic from rind and flesh of Salak Pondoh was higher compared to acarbose as a positive control. Inhibitory activity of 1% Acarbose in this research was 2.08%. This value is lower compared to the research conducted by Pujiyanto and Ferniah (2010) which reached 8.5 %. In addition to higher than acarbose, α-glycosidase inhibitor activity of isolates Kt-E and Dm-A1 was also higher when being

Table 2. Characteristic morphology of colonies and cell isolates of endophytic bacteria rind and flesh of Salak Pondoh.

Isolates	Colony Morphology				Cell Morphology	
	Color	Form	Margin	Elevation	Gram	Shape
Dt-A	Yellow	Round	Intact	Curved	-	Bacillus
Dt-B	Yellow	Irregular	Intact	Bloated	-	Coccus
Kt-E	White	Similar roots	Splitting	Crateriform	+	Bacillus
Kt-I	White	Irregular	Swam	Align	+	Bacillus
Dm-A1	White gray	Irregular	Wavy	Hill	-	Coccus
Dm-A2	White yellowish	Irregular	Wavy	Align	-	Coccus

compared to the α-glycosidase inhibitor activity of endophytic bacteria isolates of pare plant (*Momordica charantia* L.) that ranged between 16.1 -27.4% (Pujiyanto et al., 2010) and from isolates of black bean endophytic bacteria (*Castonospermum austral*) ranging from 0.62-23.31% (Peng et al., 2014).

Table 3. The activity of the α-glycosidase inhibitor from endophytic bacterial isolates from the rind and the flesh of SalakPondoh.

Isolates of endophytic bacteria	Inhibitory activity (%)
Dt-A	18.03
Dt-B	21.57
Kt-E	62.95
Kt-I	19.25
Dm-A1	30.79
Dm-A2	6.14
Acarbose 1%	2.08

Activity of α-glycosidase inhibitor of isolates Kt-E and Dm-A1 was also higher when compared with the activity of α-glycosidase inhibitors of other microbial endophytes, such as from endophytic actinomycetes isolates of Brotowali plant (heart-leaved moonseed/Tinosporacrispa) ranging between 5.64-11.01% (Pujiyanto, 2012) and from endophytic actinomycetes isolates of rhizome (*Curcuma xanthorrhiza*) with highest inhibition was 5.25% (Irawan, 2009). Later, when compared with α- glycosidase inhibitor activity from endophytic fungal isolates of Ongkea (*Mezzetia parviflora* Becc.) ranging between 18.48 - 42.56% (Hikmal et al., 2015), α- glycosidase inhibitor activity of isolates Kt-E was higher.

Identity of endophytic bacteria based on 16S rRNA

Identifikasi bakteri endofit penghasil inhibitor *a-* glukosidase dari kulit dan daging buah salak pondoh dilakukan berdasarkan sekuen gen penyandi 16S rRNA.

The encoding genes of 16S rRNA from the 6 isolates were successfully amplified with a PCR product size of 1,300 bp (Figure 3) and sequnce of 16S rRNA gene of the isolates in general shared 92-98% similarities with the data from the GenBank (Table 4).

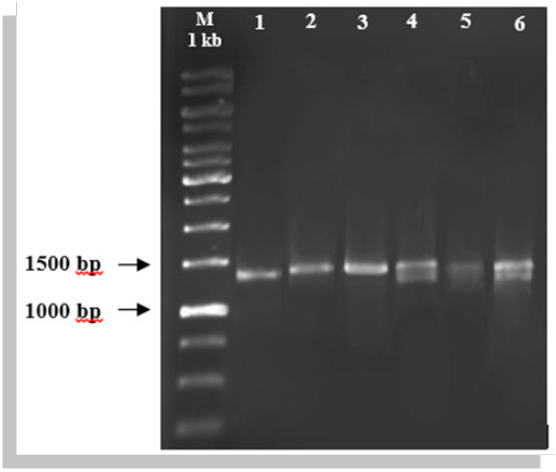

Figure 3. Electropherogram of amplicon of 16S rRNA gene isolates Dt-A (1), Dt-B (2), Kt-E (3), Kt-I (4), Dm-A1 (5), and Dm-A2 (6). M: DNA Marker 1kb (bp): 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 5000, 6000, 8000, 10000.

Dt-A and Dt-B isolates were identified as Xanthomonas genus, with similarity percentage of 97% and 98%, respectively. According to Soudi et al. (2011) and Trebaol et al. (2000), genus Xanthomonas has spherical colonies, yellow-coloured, glossy, slimy, gram-negative, and bacillus-shaped or short bacillus (coccobacillus). These morphological characteristics suit the majority of morphological characteristics of Dt-A and Dt-B isolates. Zheng et al. (2006), in one of his study, stated that one of the species of the Xanthomonadaceae family, namely *Stenotrophomonas maltrophilia* or Xanthomonas can produce α-glycosidase inhibitory compounds when grown in a medium containing validamycin A. α-glycosidase inhibitor compounds produced are identified as valienamine. According to Zheng et al. (2005), valienamine has a structure analogous to a-D- glucose that can bind to the active side of α- glycosidase enzyme.

The Kt-E isolate was identified as genus Paenibacillus with a percentage of similarity of 98%. According to Ingham and Jacob (2008), genus Paenibacillus has white-coloured colonies, similar to roots, split, longitudinal colonic growth in solid media, gram-positive, and bacillary. Those morphological characteristics have similarities with the morphological characteristics of Kt-E isolates. Research conducted by Nguyen et al. (2017) stated that *Paenibacillus sp*. such as *P. mucilaginosus* and *P. macerans*, can synthesize α-glycosidase inhibitor compounds when grown on a medium containing squid ink powder (containing 40% chitin and 60% protein). α-glycosidase inhibitory compound that was

Table 4. Similarity percentage of endophytic bacterial isolates producing α-glycosidase inhibitors from the rind and flesh of SalakPondoh with data on GenBank.

Isolate name	Description of isolates	Query cover (%)	E value	Ident.Similarity (%)	Accession number
Dt-A	Xanthomonas sacchari strain ICGV-4	93	0.0	97	KY882111.1
Dt-B	Xanthomonas sp. strain JS1029 Seed 19 B2Ptero	96	0.0	98	KX507153.1
Kt-E	Paenibacillus sp. SCU-B70	94	0.0	98	KJ000768.1
Kt-I	Bacillus thuringiensis CTC strain	96	0.0	98	CP013274.1
Dm-A1	Enterobacter xiangfangensis	87	0.0	92	LC095661.1
Dm-A2	Enterobacteriaceae	92	0.0	96	KY249127.1

generated allegedly is homogentisic acid (2,5-dihydroxy-phenylacetic acid, HGA). However, there is yet a further study of HGA compounds in inhibiting α- glycosidase.

Kt-I isolate was identified as genus Bacillus with a percentage of similarity of 98%. According to Muniady et al. (2011), *Bacillus thuringiensis* has characteristic of a white-coloured colony, opaque, flat elevation, irregular edges, gram-positive, and bacillus-shaped. These morphological characteristics have some similarities with the morphological characteristics of Kt-I isolates. Onose et al. (2013) stated that *Bacillus sp*., among them *B. subtilis* and *B. amyloliquefaciens*, are able to synthesize α-glycosidase inhibitor compounds when grown on sorbitol-containing media. α-glycosidase inhibitory compounds produced are identified as 1-deoxynojirimycin (DNJ). According to Nakagawa (2013), DNJ has an analogous structure to glucose but oxygen in the pyranose ring is replaced with nitrogen, so it can bind to the active side of the α-glycosidase enzyme.

Dm-A1 and Dm-A2 isolates were identified as the Enterobacteriaceae family with a percentage of similarity of 92% and 96%. The bacterial isolates Dm-A1 and Dm-A2 are indicated as potentially new species. According to Davin-Regli and Pages (2015), the Enterobacter genus has the characteristics of gram-negative morphology; in the form of bacilli or short bacilli (coccobacillus); pink colony colour, white, white grey, and yellow; and irregularly round to bumpy shaped. These morphological characteristics have some similarities with the morphological characteristics of Kt-E isolates. No previous research has suggested that Enterobacter xiangfangensis and Enterobacteriaceae bacterium, or Enterobacteriaceae family may produce α-glycosidase inhibitor compound.

Based on this finding, the a- glycosidase inhibitors of endophytic bacterial isolates of Salak Pondoh rind and flesh, particularly from Kt-E and Dm-A1 isolates have the potential to be developed, so that later they can be candidates of antihyperglycemic drugs for people with diabetes mellitus.

CONCLUSIONS

A total of 6 endophytic bacterial isolates potentially producing α-glycosidase inhibitor were isolated from the rind and flesh of the Salak Pondoh. Dt-A and Dt-B isolates are from Xanthomonas genus, Kt-E isolate is from genus Paenibacillus, Kt-I isolate is from genus Bacillus, isolate Dm-A1 and Dm-A2 are from family Enterobacteriaceae. The activity of α-glycosidase inhibitor from the isolates Dt-A, Dt-B, Kt-E, Kt-I, Dm-A1 and Dm-A2 were 18.03%, 21.57%, 62.95%, 19.25%, 30.79% and 6.14%, respectively. The results indicate that endophytic isolates from Salak Pondoh could potentially be the alternative source for hyperglycemia medication, especially isolate Kt-E from genus Paenibacillus.

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