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α-Amylase Inhibitor Activity of Endophytic Bacteria Isolated From *Annona muricata* L

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Abstract. α-amylase (α-1,4-glucan-4-glucohydrolase, EC 3.2.1.1) is an enzyme that catalyzes the degradation of starch into its monomers. Most people use medicinal plants for keeping normal level of blood glucose, for example, the *Annona muricata*. The objectives of this study are to obtain endophytic bacteria from the plant, knowing the activity of the α-amylase inhibitor of selected isolates. Endophytic bacteria are isolated from the roots, stems, and leaves of the plant have been sterilized surface and grown in NA medium. A total of 11 isolates were found to produce α-amylase inhibitor compounds. The isolates obtained were tested for their α-amylase inhibitor activity, and isolates with the highest activity tested further. Isolate DS21 show the best activity with 72,22% inhibition. The experimental design used in this research is Completely Randomized Design (RAL). The best isolates treated by a variety of carbon sources, and the best carbon source treated with various pH. The data obtained were analyzed usingAnalysis of Variance (ANOVA). The results of statistical tests show the treatment of starch and lactose has a significant effect on the production of α-amylase inhibitors ($P < 0.05$) and the pH 5 and 6,0 significantly affected the production of α-amylase inhibitors ($P < 0.05$).

1. Introduction

Diabetes Mellitus (DM) is categorized as a global disease by the World Health Organization (WHO) with the number of people in the world reached 199 million in 2009 [1]. This disease is a deadly non infectious degenerative disease with signs of increased levels of glucose in the blood due to disruption of insulin production as well as the result of obesity and life style that is less appropriate. This disease is classified into two types, namely DM type 1 and 2. Type 1 DM disease occurs due to the lack of insulin produced by pancreatic beta cells so that the amount of insulin hormone produced cannot be sufficient to convert blood glucose into intracellular glucose and type 1 DM can only be treated with insulin injectable therapy, whereas type 2 DM occurs as a result of obesity and life style less precise.

Conventional DM type 2 treatment can be performed by controlling food consumption, such as slowing the absorption of glucose by inhibiting the action of carbohydrate-hydrolyzing enzymes such

as α-amylase. Acarbose is a commercially available inhibitor which is known to inhibit a wide range of glycosidases such as α-amylase and α-glucosidase. However, the drugs available on the market are known to have side effects such as indigestion, bloating, diarrhea, and flatulence so that the utilization of natural ingredients as a natural diabetes remedy tends to be the community's choice. Soursop (*Annona muricata* L.) is known as a medicinal plant that can cure various diseases, such as treating abscesses, hypertension, liver disease, headache, and diabetes [2].

Utilization of endophytic bacteria is expected to be advantageous because its life cycle is shorter than its host plant and can be produced on a large scale by using fermentation process [3]. Carbon sources are known to affect various cultural parameters such as growth, primary metabolism, and secondary product yield. One of the secondary products of endophytic bacteria is the α-amylase inhibitor. Increased production of α-amylase inhibitors from endophytic bacteria can be done by regulating environmental factors during the fermentation process. The degree of acidity (pH) is one of the environmental factors that influence the activity of enzymes. Based on the above background, the endophytic bacteria isolation from Annona muricata L plants was conducted to determine the endophytic bacteria's ability to produce α-amylase inhibitors treated by various carbon sources and pH is expected to be an alternative source of drug DM type 2.

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2. Material and Methods

2.1. Isolation and Characterization of Endophytic Bacteria

This isolation technique uses the previous method [4] method with modification. Isolation of endophytic bacteria begins with surface sterilization by washing the surface of roots, stems and *Annona muricata* L leaves using water to flow until the soil and dirt disappear. The sample was then immersed in 1% sodium hypochlorite solution for 2min, then the solution was discarded and immersed in 1% more hypochlorite solution for 6 minutes. After immersion of the second 1% sodium hypochlorite solution, the sample was immersed with 70% alcohol for 1 minute. The last stage of the sample was soaked with sterile aquades for 2 minutes and repeated twice. Samples that have been soaked with aquades, the surface is sliced and crushed aseptically, then placed on the NA medium. Then incubated at room temperature for 24 hours. The sterile distilled water used to rinse the sample is used as the control captured on the NA medium. The growing colony is observed in shape, color, surface, gram, and cell shape under a microscope. Each of the different colonies is purified and the pure isolates are stored. .

2.2. Alpha-amylase inhibitor assay

To produce α-amylase inhibitors, endophytic bacterial isolates were grown in a liquid medium, according to the previous method [5] containing 0.1% soluble starch, 0.5% peptone and 0.15% yeast extract (pH 7) in a 100 ml Erlenmeyer. The culture was grown in 20 ml of the liquid medium then incubated for three days with agitation of 120 rpm at room temperature. The bacterial cell was then separated by centrifugation at 3500 rpm for 15 minutes and the supernatant obtained was tested for its inhibitory effect on α-amylase activity [6]. The activity of the α-amylase inhibitor of each sample was analyzed by the modified Bernfeld method as described below. A 0.5% starch solution was prepared by dissolving 0.25 grams of starch into 50 ml of aquadest. A total of 0.5 units/ml of the α-amylase enzyme were dissolved into phosphate buffer pH 6.9 [7]. Then, 0.5 ml of the sample was reacted with 0.5 ml of the α-amylase enzyme. After 10 min incubation at 25 ° C, 1 ml of 0.5% starch solution was added and then incubated at 25 ° C for 10 min. The same is done for the

control, where 0.5 ml of the sample is replaced by the liquid medium. After incubation for 10 min, the reaction was stopped by adding 2 ml of dinitrosalicylic acid reagent (DNS) into the control and the sample, then heated using a bath for 5 minutes. The recorded absorbance using spectrophotometer in the 540 nm [8] . The inhibition of the enzym was calculated by the formula [9]:

$$\% \text{ inhibition} = ((K-S))/K \times 100\%$$

(K: the controlled absorbance of the enzyme + substrate + liquid medium, S: controlled absorbance of enzyme + substrate + inhibitor).
After calculating the percentage of α-amylase inhibitor, an endophytic bacterial selection was performed by taking into account the results of the α-amylase inhibitor test, the highest percentage of the isolates used for the optimum test of the α-amylase inhibitor.

2.3. Determination of Optimum Production Time
The production curve is used to determine the optimum time of enzyme production used to produce large quantities of α-amylase inhibitors. The selected isolates were grown in 100 ml of production medium according the previous method [5] contained 0.1% soluble starch, 0.5% peptone and 0.15% yeast extract (pH 7) were sterilized. A total of 1 ose of isolates were inoculated into the production medium and incubated at room temperature with a speed of agitation of 120 rpm with a rotary shaker. Every 4 hours for 36 hours, a culture of 5 ml, 2 ml to calculate the activity of inhibitorα-amylase and 3 ml again used to measure the growth of endophytic bacteria [10]. Starter Creation Starter is made by growing 1 ose of bacteria isolates that have been rejuvenated in the liquid medium contains 0.1% soluble starch, 0.5% peptone and 0.15% yeast extract (pH 7) of 20 ml [5]. The culture was incubated at room temperature with an agitation of 120 rpm until OD reached 0.5 at λ 600 nm. This culture is used as a starter [11] .

2.4. Effect of Various Carbon Sources on Production of α-amylase Inhibitors.
The selected isolates were grown in 20 ml of production medium according previous research [5] containing 0.1% soluble starch, 0.5% peptone and 0.15% yeast extract (pH 7) in a 100 ml Erlenmeyer. As a treatment, the starch component is replaced with other carbon sources such as sucrose, maltose, and lactose [4]. An 1% starter with 0.5 O OD was inoculated into the production medium and incubated at room temperature with agitation rates of 120 rpm for 32 hours. At the end of the fermentation, the centrifugal culture and the obtained granulation are used for the test of α-amylase inhibition activity [8]. Absorbance was measured with a wavelength of 540 nm using a double-beam UV-VIS spectrophotometer [12].

3. Result and Discussion

3.1. Isolation of endophytes
In this study, 11 isolates of endophytic bacteria were isolated from *Annona muricata* L, 4 isolates from roots, 5 isolates from stems, and 2 isolates from leaves. Endophytic bacterial isolates derived from the roots were coded AS (roots) or AG (roots), the stems were coded BS (stem of the incision) or BG (stem), and the leaf section was coded DS (leaf cut) or DG (leaf crushed). In general, the root section is the most heavily colonized by endophytic bacteria compared to other plant parts [13]. However, the stem part of the plant is also a part that is heavily colonized by endophytic bacteria especially in the xylem vessels. This is because endophytic bacteria colonize plant tissue through a gap or wound

formed when the lateral root or root elongation zone develops and then spread to other parts of the plant, i.e., stems and leaves through the carrier network [14]. The isolation and characteristic of endophytic bacterial isolates isolated from *Annona muricata* L can be seen in Table 1.

Table 1. The characteristic of endophytic bacterial isolates from *Annona muricata* L

Parts	Code	Morphology of colony			Morphology cell	
Plant	Isolate	shape	colour	surface	shape	Gram
Root	AG11	Irregular	White	glistening	Monobacil	+
	AG21	Circular	White yellowish	glistening	Monococcus	-
	AG22	Circular	White	glistening	Monobacil	-
	AS11	Irregular	White	glistening	Monobacil	-
Trunk	BG22	Circular	White yellowish	glistening	Monobacil	-
	BG23	Irregular	White	glistening	Streptococcus	-
	BG24	Irregular	yellow	glistening	Monobacil	-
	BG25	Circular	White	glistening	Monobacil	-
	BS21	Irregular	White	glistening	Monobacil	-
Leaf	DG11	Circular	White yellowish	glistening	Monococcus	-
	DS21	Irregular	White	glistening	Monobacil	-

The α-amylase inhibitor test was used as a preliminary test to determine the isolates with the best inhibitory activity. This test was performed on 11 isolates obtained from the previous stage shown in Table 2.

Isolate	Inhibition (%)
AG11	28,86
AG21	58,20
AG22	33,11
AS11	64,47
BG22	49,81
BG23	68,19
BG24	34,08
BG25	67,85
BS21	48,70
DG11	27,21
DS21	72,22

Based on the results of the inhibition activity test showed that the eleventh isolates had considerable potential in producing the α-amylase inhibitor compound and found one of the best isolates of a DS21 isolate. The selection of isolate DS21 as isolate used for the further test was based on an activity of inhibition of isolate is highest compared to 10 other isolates, that is 72,22%. The colony and DS21 isolate cell form are presented in Figure 1.

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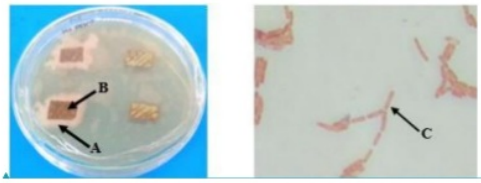

Figure 1. The morphology colony of DS21 isolate

3.2. Production of α-amylase inhibitor

The determination of optimum production time was done by making the growth curve of isolate of DS21 and testing its inhibition ability at each sampling point. The growth curve and the percentage of DS21 inhibition isolates are presented in Figure 2. The optimal inhibition activity occurred at 32 hours with inhibition activity of 60.99%. The decrease in inhibition activity and growth curve occurred at 36 hours, so the incubation process was stopped. Based on the research results, the 32nd hour is a stationary phase of bacteria. The stationary phase is the phase in which the number of live and dead bacterial populations is balanced because of the decrease in a number of nutrients in the growth medium so that at the same time bacteria produce secondary metabolites as a form of defense. According tothe previous method [15], low molecular weight enzyme inhibitors released in the culture filtrate are secondary metabolites with unclear function in microbial cell growth so further research is needed because in this study the linkage between enzyme inhibitor production and bacterial growth is not explained.

Figure 2. Growth curve and inhibition activity of isolate DS21 with 36 hours incubation period.

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Based on the results of the Analysis of Variance (ANOVA) showed that carbon starch sources affect the value of inhibition compared with other types of carbon sources. The existence of influence on Analysis of Variance (ANOVA) then performed a further test by using Tukey test (BNJ) to know whether there are different treatment pair that real. The treatment of starch and lactose significantly affected the production of the α-amylase inhibitor shown in Figure 3.

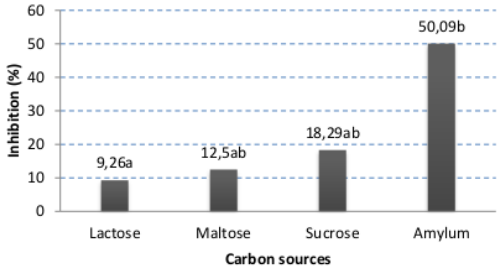

Figure 3. The activity of α-amylase inhibitors in various carbon sources.

Treatment of various pH in this study aims to determine the best pH for the production of α-amylase inhibitors. Based on the results of the Analysis of Variance (ANOVA) showed that the pH 5 treatment had an effect on the value of inhibition compared with another pH. The best percentage of inhibition at pH 5 was 27,13%, followed by pH 8 (15,85%), pH 7 (12,26%), and pH 6 (12%). There is a significant difference in the Analysis of Variance (ANOVA) then the further test using Tukey test (BNJ) to determine whether there are different treatment partners are real. Treatment of pH 5 and 6 have a significant difference in the production of α-amylase inhibitors. This inferior high value of the inhibition is believed to be derived from the α-amylase inhibitor compound produced by the isolate DS21 because of initial pH control on the medium used in the test reaction composition for the control and growth medium of endophytic bacteria. The graph of activity of α-amylase inhibitor on some pH can be seen in Figure 4.

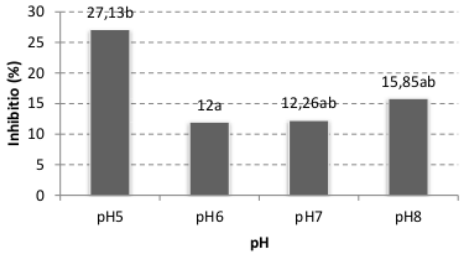

Figure 4. The activity of α-amylase inhibitors at various pH.

Based on the results of statistical analysis shows that the pH 5 treatment can increase production of α-amylase inhibitors. This is thought to be caused by the acid tolerance response by bacterial isolates DS21. Based on Gram staining, DS21 isolates belong to the Gram-negative group of bacteria. Most microorganisms grow well at neutral pH [16], but Gram-negative bacterial properties are less resistant to acidic conditions so that growth medium with pH 5 is considered to be a less favorable environment for endophytic bacteria. This unfortunate condition is thought to be a trigger of secondary metabolite synthesis of α-amylase inhibitor compounds. According to [17], secondary metabolites are produced when environmental conditions are less favorable as a form of self-defense of an organism.

4. Coclusion

Based on the result of the research, it can be concluded that the isolation of endophytic bacteria from *Annona muricata* L was obtained by 11 isolates which have potential to produce α-amylase inhibitor compound, i.e. AG11, AG21, AG22, AS11, BG22, BG23, BG24, BG25, BS21, DG11, and DS21. Isolates with the highest inhibition activity were isolated of DS21 with a percentage of inhibition 72,22% so used for a further test. Treatment of carbon source of starch and pH 5 can increase production of α-amylase inhibitors.

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