The hydrolysis of carbohydrate-based local raw materials as natural sweeteners by extract powder enzyme *Aspergillus awamori* KT-11 from wheat bran

The hydrolysis of carbohydrate-based local raw materials as natural sweeteners by extract powder enzyme *Aspergillus awamori* KT-11 from wheat bran

R Melliawati ,Nuryati and A F Mahmudah

Research Center for Biotechnology -LIPI
Jln. Raya Bogor Km 46 Cibinong, 16911, Indonesia

Email: ruthmell2000@yahoo.com

Abstract.. *Aspergillus awamori* KT-11 is a proven potential mold produces the amylase enzyme complex, particularly amylase and glucosidase. These enzymes hydrolyze the starch being sugar. Food and beverage industry began extensive use of liquid sugar because it has several advantages. Liquid sugar is raw materials of starch in nature Indonesia. This purpose of this research is to extract powder enzyme *Aspergillus awamori* KT-11 from wheat bran against 4 types of raw material-based local carbohydrate to produce natural sweetener (liquid sugar).The raw material used namely potato, breadfruit, yellow squash, and bananas. Enzyme activity produced from mold *Aspergillus awamori* KT-11 on wheat bran of 186,9835 U/ml, reducing sugar of 9.775 mg/ml and protein levels is 48.981 mg/ml. The sweetener from natural potato starch, breadfruit, yellow squash, and banana produces different percentages. The highest percentage of banana flour produced at 117.938 mg/ml (concentration of flour 20%, enzyme 15% and incubation time for 3 days) and lowest on yellow squash (6.563 mg/ml). The result shows that TLC from potato flour, breadfruit and bananas are produced of glucose and lactose, is from flour yellow squash generated fructose.

1. Introduction

Indonesia is an agricultural country with abundant natural resources. One of the commodities that have not been used well is tubers [1]. Developed countries have long used this food as a processed product of high nutritional value and economically has a large market opportunity. High carbohydrate content in tubers makes it one of the most potent biological resources to be developed, especially in the effort to realize national food security [2]. Types of tubers are very diverse as well as carbohydrate content, including cassava, sweet potatoes, cone tubers, taro, black potatoes, arrowroot tubers, and others. In general, the sweetness of the tubers is obtained through the process of decomposing carbohydrates (starch) by the amylase enzyme into sugar. Sugar produced from the decomposition process is glucose, sucrose, and fructose. This type of sugar determines the sweetness of each type of tuber. The sweet taste in these tubers correlates with the amount of sugar available, especially the reducing sugars such as glucose and fructose [3].

Liquid sugar starts to be widely used by the food and beverage industry because it has several advantages including liquid sugar not crystallized, easier to process because it is more soluble, more practical, and has a more attractive appearance when compared to sugar in general. High market demand makes the rate of imports of liquid sugar even higher, according to [4] in 2015 the production of liquid sugar was 2.9 million tons while the demand for liquid sugar in the market was 5.9 million tons. It is expected that Indonesia is able to meet the needs of liquid sugar without having to import,

given the raw material for liquid sugar is starch / carbohydrates which are widely found in Indonesian nature, such as cassava, Kimpul, Taro, Potatoes, Breadfruit, Bananas, Sweet potatoes, Porang, Gadung and others.

Starch is a glucose homopolymer when it is hydrolyzed being glucose. According to [5], the making of liquid glucose by enzymatic hydrolysis occurs through two stages, namely Liquification and saccharification. Both of these processes are influenced by various factors, such as enzyme concentration, substrate concentration, pH, temperature, processing time and the presence of inhibiting compounds[6]. Liquid glucose production from starch material can be produced optimally if the optimum condition production has obtained. [7] reported that the production of liquid sugar using Gadung tuber starch by using the hydrolysis process of amylase enzyme can produce liquid sugar 10.39% (b/v) with a dextrose equivalent (DE) 34.64%. The low concentration of sugar and the DE value produced in the study can be caused by the hydrolysis process only using the amylase enzyme so that not all starch fractions will be hydrolyzed. In the process of hydrolysis of starch by using only the amylase enzyme, in general, it will only hydrolyze the α-1,4glycosidic bonds, while the α-1,6 glycosidic bond cannot be hydrolyzed, so it still produces a dextrin product. To increase the concentration of sugar and DE in the starch hydrolysis process, it is necessary to carry out a saccharification process using the amyloglucosidase enzyme. The utilization of α-1.6 glycosidic bond from this enzyme will hydrolyze the DE to glucose.

Research Center for Biotechnology LIPI has *A. awamori* KT-11 that has been tested for its ability to hydrolyze starch. This isolate is capable of producing complex amylase in solid media using wheat bran. The mold is known to produce three types of amylase, namely α-glucosidase, α-amylase, and glucoamylase. From α-amylase, three types are obtained, namely, Amyl I, II and III, of which two of them (Amyl II and III) are able to hydrolyze raw starch [8]and two types of glucoamylase (GA I and II), has the ability to hydrolyze raw starch simultaneously [8]. When α-amylase and glucoamylase work together, the act of breaking down starch increases by three times compared to if each enzyme works alone [9]. This is caused also because glucoamylase from mold has the ability to cut the α-1,6 glucoside chain from starch or that means it has a high debranching activity [10].

High carbohydrate content in tubers can be a potential biological resource to be developed. Therefore in this research yellow squash, potatoes, breadfruit, and banana flour are used as substrates for making natural sweeteners (liquid sugar). The purpose of this study was to hydrolyze four types of flour tubers to make natural sweeteners (liquid sugar) with the help of complex enzymes from *Aspergillus awamori* KT-11.

2. Materials and Methods

2.1. Microorganism

Aspergillus awamori KT-11 is one of microorganisms collection of the Research Center for Biotechnology LIPI, which it is has been tested for its ability to hydrolyze starch. *Aspergillus awamori* KT-11 was transferred to PDA media and incubated for 5 days at room temperature.

2.2. Making flour

Yellow squash and fresh potatoes are cleaned and peeled, weighing each weight, then sliced tubers with a thickness of about 1 mm. Then dried in a drying oven at 60° C for 48 hours to dry. Then puree with a blender and sifted with a 200 mesh sieve. Yellow squash and potatoes ready for use. Banana and breadfruit flour is obtained from Gunung Kidul (Putri 21)

2.3. Making powder enzymes in wheat bran media by Aspergillus awamori KT-11

Wheat bran Dry as much as 100 grams plus 1% K_2HPO_4, 1% KH_2PO_4, 2.5% Technical ZA, and 1% Soluble Start, then added 100 ml of distilled water, stirred and sterilization at 121º C for 15 minutes. Enter the suspension of *Aspergillus awamori* KT-11 (5 mL of sterile water was put into a test tube containing *Aspergillus awamori* KT-11 and dissolved)into the Wheat bran, stirred until suspension is completely mixed, then the pan is closed again using sterile paper and incubated at room temperature for 7 days, then dried in an oven at 50˚C for 24 hours, mashed into enzyme powder ready for use.

2.4. Making enzyme extracts (Crude enzymes)
Enzyme powder as much as 1 gram was dissolved in 5 mL of citrate-phosphate buffer pH 4.8. shaken for 2 hours at 4˚C. Then centrifuged for 10 minutes (2 times) at a speed of 10,000 rpm. The filtrate is taken and filtered again using filter paper, the result is a crude enzyme.

2.5. Fermentation process
In this study, there were two types of treatment, namely fermentation time and flour substrate concentration used. The fermentation time is 3 days and 4 days, while the concentration of flour is 15% and 20%, the crude enzyme concentration used for this research is 15%. The initial step, each flour sample weighed as much as 0.75 grams (15% concentration) and 1 gram flour (20% concentration) then each adds 5 ml of pH 4.8 citrate-phosphate buffer. The mixture of flour and buffer is then heated in the gelatinization process. Then each tube is inserted into the water bath (Memmert) with a temperature of 60° C until the temperature is stable and then inserted 0.75 ml of the crude enzyme and incubated for 3 days and 4 days, then each tube is heated in boiling water to stop the enzyme activity. The sample was transferred in a centrifuge tube and centrifuged (Centrifuge Kubota 6200) at a speed of 3500 rpm at 4˚C for 10 minutes (two rounds). The resulting filtrate is then transferred into a tube and stored in cold temperatures.

2.6. Determination of enzyme Activity
The test tube containing 0.02 grams of Soluble Starch was added with 0.4 ml of pH 4.8 citrate-phosphate buffer, then heated to boiling water for the gelatinization process. Then placed on a thermoline (Thermoline Type 16500 dry bath) temperature of 60° C, then added 0.1 ml of crude enzyme. As a substrate control, 0.02 grams of soluble starch, added 0.5 ml of citrate-phosphate buffer pH 4.8 (without adding crude enzyme), whereas for enzyme control it contained only 0.4 citrate-phosphate buffer pH 4.8 and 0.1 ml of the crude enzyme, then incubated for 1 hour. Enzyme activity is stopped by heating in boiling water. futhermore 0.5 ml of distilled water was added to each test tube, added 1 ml of Nelson A and B reagent mixture and heated for 20 minutes in boiling water, then cooled using running water or ice until cool. Then added 1 ml of C reagent, shake using by vortex then added distilled water to 25 ml. Measured Optical Density (OD) on a spectrophotometer (Genesys 10S UV-vis) with a wavelength of 500 nm. In this test using a blank that is 1 ml of distilled water, which is given the same treatment as the enzyme sample.[11]. One unit of enzyme activity is equivalent to 1 mg of reducing sugar per ml in the above conditions.

2.7. Determination of reducing sugar
Each dilution level (10 times, 100 times, 500 times and 1000 times) was taken 1 ml (Triple of the tube), then added 1 ml of Nelson A and B reagent mixture, after which it was heated for 20 minutes, then cooled using running water or ice until it's cold. Then added 1 ml of C reagent, in the vortex and then treated to 25 ml using distilled water. Measured Optical Density (OD) on a spectrophotometer with a wavelength of 500 nm. In this test using blanks that is 1 ml of distilled water, which is given the same treatment as the previous enzyme sample.[11].

2.8. Determination of protein content[12]
Preparing the enzyme extract with 10 times dilution, then enzyme filtrate was measured the Optical Density (OD)using a UV-vis spectrophotometer with a wavelength of 280 nm. OD (Optical Density) measured in the calculation as follows:

$$\text{Protein level} = \text{OD} \times \text{dilution level} \times 1.45 \quad (1)$$

3. Results and Dicussion
Enzymes made from wheat bran obtained enzyme activity of 186.9835 U / ml (Table 1). The glucoamylase enzyme activity obtained from cassava peel medium was weaker than enzyme activity on cassava skin substrate plus mineral (452 U / mL)[13]. But it is greater than the enzyme activity of

Canna flour substrate of 62 U / ml [14]. Meanwhile, the reducing sugar obtained was 9.775 mg/ml, the result was greater than the reducing sugar of canna flour at (1.59 mg/ml) and motecaf flour as much as 1.93 mg/ml [14]. The resulting protein content was 48,981 mg/ml, this result was greater than the protein content obtained from cassava skin substrate which was added with minerals, as 12,296 mg/ml (through oven drying) and 34,858mg / ml (freeze-dry drying) [13]. Wheat bran is a grain leather waste that still contains 34% starch [15], while cassava peels contain 44-59% starch [16]. This can explain that the results of enzyme activity on cassava skin is greater than wheat bran because cassava peels contain higher starch than wheat bran, so *Aspergillus awamori* KT-11 will release more enzymes to hydrolysis the starch.

Table 1. Results of enzyme activity, reducing sugars, and proteins from crude enzymes *Aspergillus awamori* KT-11 on wheat bran media.

Enzyme Activity (U/ml)	Reducing sugar (mg/ml)	Protein (mg/ml)
186,9835 U/ml	9,775 mg/ml	48,981 mg/ml

At the same flour concentration (15%) of the four samples gave different results (Figure 1), so the fermentation time affected the reducing sugar produced. Increased fermentation time, higher yields are obtained, this is because the chance of enzymes to hydrolyze starch becomes longer, so that the sugar formed will be more Potato flour produces the highest reducing sugar that is equal to 103,975 mg/ml (15% flour concentration with 4 days fermentation time), then the second order is banana flour which is 98,975 mg/ml and the third is breadfruit flour 93,25 mg/ml. In Yellow squash, the lowest yield is 8 mg/ml (fermentation 4 days). This is related to the carbohydrate content in Yellow squash, which is the lowest carbohydrate content compared to the other three samples.

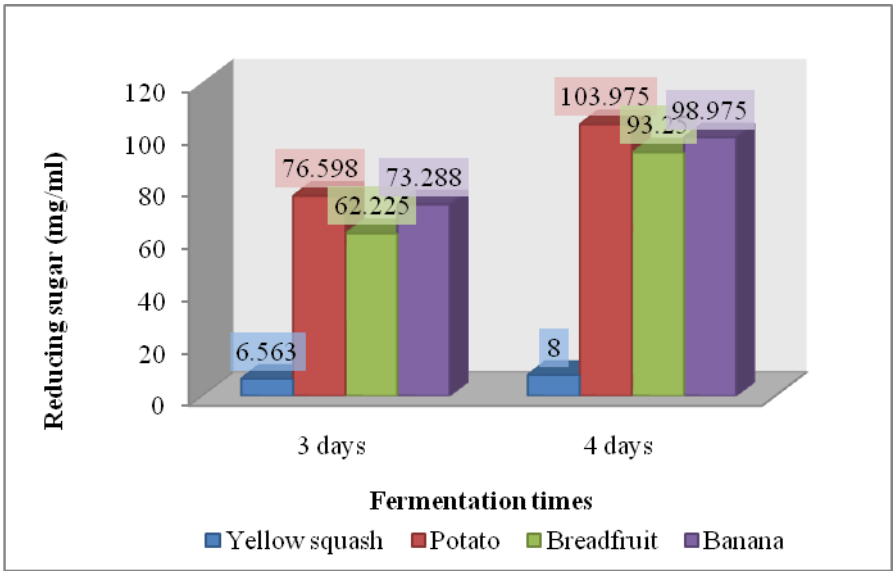

Figure 1. The results of reducing sugar in all four types of flour with 15% flour concentration were fermented for 3 and 4 days

At a concentration of 20% flour and fermentation time 3 days, reducing sugar was obtained was higher than 4 days (Figure 2) This is not only because of the length of fermentation but the starch/carbohydrate content in flour also affects the yield of reducing sugars. Banana flour has the highest yield of reducing sugar that is equal to 117.938 mg/ml, the second order is potato flour which is 103.975 mg/ml, the third is breadfruit flour of 98.875 mg/ml while the lowest reducing sugar is produced by yellow squash of 8.885 mg/ml (concentration 20% flour with a fermentation time of 3

days). The reducing sugar produced is related to the starch/carbohydrate content found in the sample.[17] reported that bananas contain carbohydrates in 100 grams of wet weight by 21 grams, potatoes contain 19.2 grams, breadfruit is 9.2 grams (young breadfruit) and 28.2 grams (old breadfruit), and yellow squash at 6.6 grams. The carbohydrate sequence contained in the sample is in line with the reducing sugar produced by each sample.

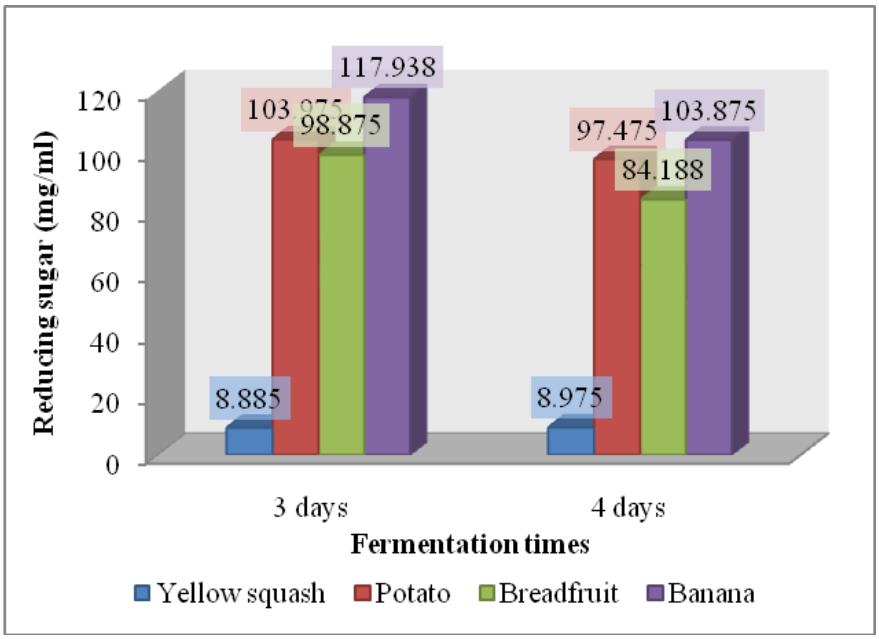

Figure 2. The results of reducing sugar in all four types of flour with 20% flour concentration were fermented for 3 and 4 days

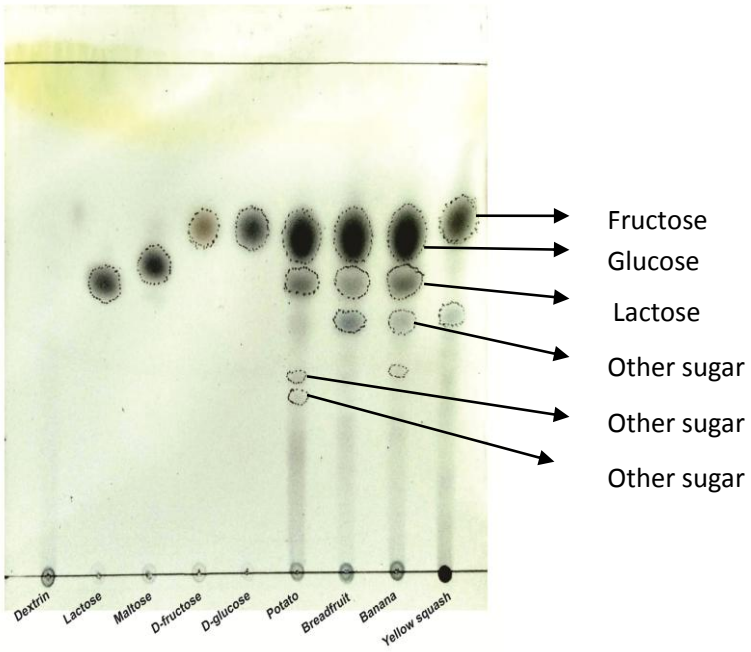

Figure 3. The result of TLC from 4 kinds of sample of fermentation result by *Aspergillus awamori* kt-11

Table 2. The analysis of the type of sugar fermented from each sample

Standard/ Sample	Rf value	K Information of sugar type
Standard :		
Dextrin	0	
Laktose	0,575	
Maltose	0,6	
D-Fruktose	0,688	
D-Glukose	0,675	
Sample:		
Potato	0,675	D-Glukose
	0,575	Laktose
	0,388	
	0,35	not in standard
		not in standard
Breadfruit	0,675	D-Glukosa
	0,575	Laktosa
	0,5	
		not in standard
Banana	0,675	D-Glukose
	0,575	Laktose
	0,5	not in standard
	0,4	not in standard
Yellow squash	0,688	D-Fruktose
	0,5	

TLC results from each sample and standard sugar (Table 2), show that each sample has a different Rf value. Potatoes showed 4 spots, breadfruit 3 spots, and bananas 4 spots. The spots from the three samples are 2 spots, each of which has the same Rf value as the Lactose and glucose. While the yellow squash produces 2 spots, one of the spots is the same Rf values as fructose. Lactose is included in a disaccharide sugar molecule, while glucose and fructose are simple sugars. Lactose can be broken down into simple sugars namely galactose and glucose. Fructose has a sweeter taste than ordinary sugar (1.7 times sweeter than ordinary sugar) and every gram of fructose contains 3 calories. Fructose is a sugar that is safe for consumption by people who suffer from diabetes, because the amount of calories is low from sucrose, so it can be consumed without worrying about weight gain.

Table 3. Fermented sugar sweetness levels

Sample	Volume (mL)		Sweetness Level (Brix)	
	Before fermentation	After fermentation	Sweet early	Concentrated 3 times
Potato	100	106	12	35
Breadfruit	100	92,5	12	35
Banana	100	100	13	40
Yellow squash	-	-	11,2	-

Sweetness levels of the four samples were not significantly different (Table 3), but bananas had a high sweetness level compared to the other samples, namely 13 brix, while the sweetness level of potato flour and breadfruit were 12 brix and 11.2 brix of yellow squash. This is because bananas have

a higher carbohydrate content than the other three samples. When viewed from the results of the analysis of reducing sugars from bananas, the highest yield was 117.938 mg/mL therefore the highest sweetness of the results of hydrolysis of banana flour was obtained.

4. Conclusion

In wheat bran media, the enzyme activity of *Aspergillus awamori* KT-11 reached 186,9835 U/mL, protein content was 48,981 mg/mL and the reducing sugar was 9,775 mg/mL. Banana flour produces the highest reducing sugar (117,938 mg/mL) at a concentration of 20% with a duration of 3 days fermentation. Yellow squash flour produces the lowest reducing sugar (6.563 mg/mL) with flour concentration 15% and duration of fermentation 3 days. TLC results show that potato flour, breadfruit, and banana produce glucose and lactose, while fructose is produced from yellow squash flour. Bananas had a high sweetness level compared to the other samples, namely 13 brix. The tubers can be developed for liquid sugar production.

5. Refferences

[1] Husna, Novita, Rohaya S 2013 The anthocyanin content and antioxidant activity of fresh purple sweet potato and its processed products *J.Agritech.* **33**(3) 296-302
[2] Ambarsari I, Sarjana, Choliq A 2009 Recommendations in Determining Standard Quality of Sweet Potato Flour *J.Standardization* **11**(3) 212 – 21
[3] Al-Kayyis H, Susanti H 2016 Comparison of the Somogy-Nelson and Anthrone-Sulphate Methods in Determination of Reducing Sugar Levels in Cilembu Bulbs (IpomeaBatatas L.) *J.Science and Community Pharmacy* **13**(2) 81-89
[4] Prasetyo, Didik 2017 Towards a Competitive Sugar Industry. National Seminar
[5] Risnoyatiningsih, Sri 2011 Hydrolysis of starch Saccharides from sweet potatoes using enzyme UPN Veteran East Java: *J. Chem. Engineering* **5**(2) 417-424
[6] Machovic, M. & Janecek, S 2007 Amyloliticenzyme : Types, structures, and specificities. In Polaina, J danMacCabe, A.P. Industrial Enzyme. Springer: Netherlands
[7] Parwiyanti, Filli P. Renti A 2011 Chemical and physical properties of liquid sugar from starch of tuber(DioscoreahispidaDents*) J. Teknol.And Food Industry* **17**(2) 171-176
[8] Anindyawati, T., R. Melliawati, K. Ito, M. Iizuka, and N. Minamiura 1998 Three Different Types of α-Amylases from AspergillusawamoriKT-11: Their Purification, Properties and Spesificities *Biosci. Biotechnol.Biochem.* **62**(7) 351-1357
[9] Ueda, S 1981 Fungal glucoamylase and raw starch digestion *Trends Biochem. Sci*. **6**(3) 89-90
[10] Yamamoto, T 1988 The amylase research society of Japan. Handbook of amylases and Related enzymes. Their sources, isolated methods, properties and applications.Pergamon Press, Oxford.
[11] Nelson, N 1941 A Photometric adaptation of the Somogy method for the determination of glucose.*J. Biol. Chem* **153** 375-380
[12] Hanson, R.S and J.A.Philips 1981 Manual of methodsfor general bacteriology. American Society for Microbiol. Washington DC. p.359-360
[13] Perwitasari, U., Nuryati., Ruth, M., &Yopi 2017 Glucoamylase Production by AspergillusawamoriKT-11 In Solid-State Fermentation Using Cassava Peel as Substrate *Annales Bogorienses* **21**(1) 21-28
[14] Melliawati, R., &Nuryati 2013 Selection of Bulbs and Cereals Flour as Alternative Raw Materials for Making Amiloglucosidase Enzymes by Aspergillus AwamoriKT-11 *Proceedings of the National SeminarXVI,Chemistry in Development* 241-246.pp
[15] Palmarola-Adrados, B, Borska, P C, Galbe, M, and Zacch, G 2005 Ethanol production from non-starch carbohydrates of wheat bran *Biores. Technol.* **96** 843–850
[16] Richana N 2013 Know the potential of cassava & sweet potatoes.Nuansacendikia. Bandung
[17] Republic of Indonesia Ministry of Health 1997 Nutrition Guidelines on Food Ingredients. Jakarta: Directorate General of Public Health. Directorate of Community Nutrition

Acknowledgements
Thank you to Dr. Yopi as the head of the biocatalyst and fermentation laboratory who gave the opportunity to conduct this research. This research was supported by government funding of Research Center for Biotechnology, LIPI. FY 2017.