Activity Enhancement of Antioxidant Contained in Sugar Palm Fruit (*Arenga pinnata* Merr) Through Solid State Fermentation by *Aspergillus oryzae*

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Abstract. It has been widely known that sugar palm fruit (*Arenga pinnata* Merr) contains galactomannan that has antioxidant activity. Antioxidants are compounds that can inhibit free radical formation during oxidation reactions in the body that can cause disease. Antioxidant compounds are commonly found in natural sources. Some research has been conducted to increase antioxidant activity in natural sources through solid state fermentation by some fungi with natural sources as substrate. This research aims to increase antioxidant activity in sugar palm fruit through solid state fermentation by *Aspergillus oryzae*, by optimizing fermentation condition parameters using Central Composite Design method. The parameters were sugar palm fruit substrate (50–300 g), *Aspergillus oryzae* inoculum (10–30 mL/100 g substrate), and $(NH_4)_2SO_4$ concentration (0–1% w/v). The fermentation was conducted at 37 ^oC for 12 days, and followed by extraction using 96% ethanol. Total Phenolic Content (TPC) was measured using reagent Folin-Ciocalteu and Total Antioxidant Capacity (TAC), expressed in IC_{50}, and was measured by DPPH method. The highest TPC was 289.69 mg GAE/g that obtained when using 300 g of sugar palm fruit substrate, 30 mL/100 g substrate of inoculum, and 0.68% w/v $(NH_4)_2SO_4$. Meanwhile, the highest IC_{50} obtained was 50 ppm when fermentation was carried out by using 300 g of sugar palm fruit substrate, 30 mL/100 g substrate of inoculum, and 0.087% w/v $(NH_4)_2SO_4$. From this result, it can be concluded that antioxidant activity of sugar palm fruit can be enhanced by *Aspergillus oryzae* through solid state fermentation method.

Keywords: Antioxidant · Aspergillus oryzae · Central Composite Design · Solid state fermentation · Sugar palm fruit

1 Introduction

Sugar palm fruit (*Arenga pinnata* Merr) is one of natural sources whose potential has not been greatly explored. Sugar palm fruit production is abundant in Toba Regency, North

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Sumatera, Indonesia. It grows best at 500–800 masl and annual precipitation of 1500–3000 mm [1]. From an area of 259 Ha of productive sugar palm, Statistics Indonesia in North Sumatera reported that the production of palm sugar reached 396.43 tonnes in 2018. On the other hand, sugar palm fruit, produced 50–100 kg/tree, has only been utilized as raw material in food industry [2]. 100 g of sugar palm fruit contains 91.8% of water, 77 kkal of energy, 0.4 g protein, 0.2 g of fat, 1.6 g of fibre, 91 mg of calcium, 243 mg of phosphor, 0.5 g of iron, and 6 g of galactomannan [3].

Galactomannans are polysaccharides composed of mannose and galactose chains. It has been utilized in textile, pharmaceutical, biomedical, cosmetics, and food industries [4–6]. It has been known that galactomannans in sugar palm fruit have antioxidant and antibacterial properties [5, 7].

Antioxidants are compounds that are capable of inhibiting oxidation reactions by free radicals so that tissue damages in our body can be prevented. Antioxidant is classified into two main classifications: enzymatic antioxidant and non-enzymatic antioxidant. Enzymatic antioxidant examples are catalase, superoxide dismutase, and glutathione peroxidase. Meanwhile, non-enzymatic antioxidant examples include carotenoid, alkaloid, flavonoid, and coumarin. All these compounds can be found in natural sources. Antioxidant compounds have been widely utilized in pharmaceutical and medicine industry because they are beneficial for preventing disease, such as cancer, when used with appropriate dose [8–10].

Antioxidant compounds can be obtained through extracting natural sources. Many studies have proven that antioxidant activities from natural sources can be enhanced through solid state fermentation using several types of fungi, such as *Rhizopus oryzae*, *Aspergillus awamori*, *Aspergillus oryzae*, *Ganoderma lucidum*, and *Pleurotus ostreatus*. The fermentation can be performed by using yellow kapok banana peel waste (*Musa acuminata balbisiana*), mung bean (*Vigna radiata*), cow pea (*Vigna unguiculata*), black lentils (*Vigna mungo*), pigeon pea (*Cajanus cajan*), lentils (*Lens culinaris*), wheat grain, flour and bran, candlenut kernel (*Aleurites moluccana*), brown and white teff grain, and rice bran as substrates [11–18].

That being said, the content and antioxidant compounds in sugar palm fruit have potentials to be increased through solid state fermentation using *Aspergillus oryzae*. The observed parameters to determine the antioxidant compound increase was total phenolic content (TPC) while the observed parameter determine the antioxidant compound activity was total antioxidant capacity (TAC). Variations of fermentation conditions optimized using Central Composite Design to observe the maximum TPC and TAC increase were the amount of sugar palm fruit substrate, concentration of inoculum *Aspergillus oryzae*, and the concentration of $(NH_4)_2SO_4$.

2 Material and Methods

2.1 Substrate Preparation

Freshly ripe sugar palm fruit was cleaned with water and drained. A preliminary study was conducted to examine the sugar palm fruit moisture content over drying time at 60 °C. Based on the result of a regression analysis, it was found that it took 5 h 30 min to produce sugar palm fruit substrate with moisture content of 60%. The data was required

to reach the moisture content of 70% when inoculum was added into the fermentation media. (Data is not shown).

2.2 Cultivation of *Aspergillus oryzae*

In this study, cultivation of *Aspergillus oryzae,* and the making of a standard curve and a growth curve followed the procedure described in [14]. The cultivation was performed in a medium potato dextrose agar (PDA). The inoculum for making the standard and the growth curves was grown in a medium yeast peptone glycerol (YPG) and incubated at 37 °C for 72 h.

2.3 Inoculum Preparation

For the fermentation inoculum preparation, 3 inoculating loops of *Aspergillus oryzae* was added into the medium YPG, and then incubated at 37 °C for 168 h, or with absorbance 1.584 at wavelength of 600 nm and cell concentration of 0.0626 g/mL. The preparation was conducted aseptically.

2.4 Standard Curve of Gallic Acid

A standard curve of gallic acid was generated by using eight variations of gallic acid concentrations ranging from 0–100 ppm. As much as 1 mL ethanol absolute was added to 0.01 g gallic acid in a volumetric flask 100 mL. Then, more of the ethanol absolute was re-added to the gallic acid until it reached the maximum mark. From 100 ppm of the stock solution, 1 mL was moved into a volumetric flask 10 mL and added with 1 mL reagent Folin-Ciocalteu. The solution was homogenized and stored for 3 min. After that, 4 mL of Na_2CO_3 1 N was added into the volumetric flask and stored for 15 min at 25 °C. The solution absorbance was measured by using a spectrophotometer at wavelength of 725 nm [19]. The gallic acid absorbance value was plotted against gallic acid concentration. Finally, the linear regression curve was generated, and the linear equation was solved. The total of phenolic was mg Gallic Acid Equivalent (GAE)/g dry extract.

2.5 Fermentation

The inoculum was moved aseptically into a fermentation flask containing the substrate according to the experiment variations shown in Table 1. The fermentation was performed at 37 °C for 12 days. The fermentation period, 12 days, was determined based on the growth curve of *Aspergillus oryzae* which showed that it reached stationary phase in 12 days [14]. Stationary phase is when secondary metabolites are produced. During the fermentation, fungi produced extracellular enzymes that released phenolic compounds from the substrate matrix, together with phenolic compounds production during secondary metabolism [20]. Optimized variations of fermentation conditions were the amount of sugar palm fruit substrate, the concentration of *Aspergillus oryzae* inoculum, and the concentration of $(NH_4)_2SO_4$ (Table 1).

Table 1. Experiment variations based on Central Composite Design method

Run	Substrate (g)	Inoculum (mL/100 g substrate)	$(NH_4)_2SO_4$ (% w/v)
1	50	10	0.5
2	300	10	0.5
3	50	30	0.5
4	300	30	0.5
5	50	20	0
6	300	20	0
7	50	20	1
8	300	20	1
9	175	10	0
10	175	30	0
11	175	10	1
12	175	30	1
13	175	20	0.5
14	175	20	0.5
15 (Control)	175	0	0

2.6 Extraction

At the end of the fermentation, 100 mL ethanol 96% was added into each fermentation flask and into a flask containing 175 g sugar palm fruit without fermentation (control), and stirred using a magnetic stirrer for 24 h at 25 °C. The extract was filtrated using a gauze and concentrated using a vacuum evaporator at pressure of 0 bar at temperature of 40 °C. The concentrated extract was used for measuring TPC and TAC.

2.7 Total Phenolic Content (TPC) Determination

TPC was determined based on a modified method [21]. As much as 0,1 mL of aliquot extract was mixed with 1 mL reagent Folin-Ciocalteu and stored to react for 3 min. Next, 300 μL Na_2CO_3 1N was added and left react for 90 min at 25 °C. The absorbance value was measured at wavelength of 725 nm. The absorbance value was next substituted into a standard curve of gallic acid. Finally, the concentration of total phenolic was obtained (mg GAE/g).

2.8 Total Antioxidant Capacity (TAC) Determination

TAC was determined through the method of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) [22]. Every 25 mg of the sample was dissolved using ethanol 96% in a volumetric flask

Table 2. Total phenolic content and total antioxidant capacity in optimum solid state fermentation condition

			Substrate (g)	Inoculum (mL/100 g substrate)	$(NH_4)_2SO_4$ (% w/v)
TPC (mg GAE/g)	C	86.06	175	-	-
	F	289.69	300	30	0.680
TAC (IC_{50}) (ppm)	C	600.91	175	-	-
	F	50	300	30	0.087

Note: C = control; F = fermentation

100 mL. The homogenized sample was next diluted until 5 variations of concentrations were obtained: 50 ppm, 100 ppm, 150 ppm, 200 ppm, and 250 ppm. As much as 2 mL DPPH 50 ppm was added to 2 mL sample of each concentration. As for blank solution, 1 mL ethanol 96% was mixed with 2 mL DPPH 50 ppm. All the solutions were incubated at 25 °C for 30 min. Then, the absorbance value was measured at wavelength of 517 nm using a spectrophotometer UV-Vis. The inhibition percentage was plotted over the concentration so that a linear regression curve was generated, and the linear equation was solved. Based on the linear equation, determine the inhibition percentage of 50% or IC_{50}. The percentage of inhibition can be calculated using the formula below:

$$\% \text{ inhibition} = \left(\frac{\text{Blank Abs.} - \text{Sample Abs.}}{\text{Blank Abs.}} \right) \times 100 \quad (1)$$

3 Result and Discussion

As seen from the data analysis shown in Table 2, to obtain maximum TPC and TAC in this study, it required the same amount of the sugar palm fruit substrate and concentration of the inoculum. With the substrate amount of 300 g and the inoculum concentration of 30 mL/100 g, the TPC was 289.69 mg GAE/g, and the TAC was IC_{50} 50 ppm. TPC of 289.69 mg GAE/g was reached with an addition of ammonium sulfate 0.68% w/v, while TAC of IC_{50} 50 ppm was obtained with an addition of optimum ammonium sulfate 0.087% w/v. The TPC of the control sample which was the extraction result of 175 g sugar palm fruit substrate without the addition of *Aspergillus oryzae* inoculum and ammonium sulfate was 86.06 mg GAE/g. Meanwhile, the TAC of the control sample of IC_{50} 601 indicated lower inhibitory activity compared to the TAC of the fermentation result.

There have been many studies that used *Aspergillus oryzae* and solid-state fermentation to enhance the total phenolic content yielded by plants. In a study conducted by [12], a sample of 5-day fermentation using wheat bran contained TPC around 3.5 times higher (1469.35 μM/g GAE) in methanol extract compared to the control sample in the study. In another study [23], 28-day solid state fermentation using *Aspergillus oryzae* and turmeric produced TPC of 374.3 mg GAE/g or 1.43 time higher than the control sample

in the study. Magro et al. [24] conducted research on optimizing potential of antioxidant compounds in lentils (*Lens culinaris* L.) by comparing the use of *Aspergillus niger* and *Aspergillus oryzae* in solid state fermentation. TPC of the extract yielded from using *Aspergillus oryzae* reached 4.27 GAE/g after 96 h of fermentation. It was 2.8 times higher than the extract produced from the fermentation using *Aspergillus niger,* and it was higher fourfold compared to the control sample in the experiment. Solid state fermentation using candlenut and *Aspergillus oryzae* done until stationary phase showed an increase of TPC as much as 23.28 times higher than the control sample in a study conducted by Limbong et al. [14]. Punia et al. [17] also examined the potential of rice bran fermentation by *Aspergillus oryzae*, and it was concluded that TPA produced was 8.83 g GAE/g dwb after 4 days. It was 8.17 times higher than the control sample in the study.

Based on the previously mentioned studies, it is visible that solid state fermentation by *Aspergillus oryzae* can increase the TPC extracted from plants. In a study performed by Ohara et al. [25], during solid state fermentation, microorganisms in the fermentation produced various hydrolytic enzymes, such as amylase, lipase, and β-glucosidase. The enzymes functioned to hydrolyze phenolic compounds in plant cells, so they could be diluted and their concentration increases in water extract. On the other hand, the benefit of the enzymes from microorganisms was not present in the control sample.

Phenolic compounds in plants function in defense mechanism and act in other biological functions, such as antioxidant activity. Bioactive compounds found in plants are closely related to antioxidant activity and TPC, and phenolic compounds contributes greatly to antioxidant activity [26]. Therefore, besides increasing TPC production, solid state fermentation also can enhance antioxidant activity. In solid state fermentation using wheat bran as the substrate, an increase in antioxidant activity in phenolic compounds extracted using methanol was visible [12]. A significant increase as much as 71.30% could be noticed after 2 days of fermentation. It was higher in comparison to antioxidant activity of 68.04% in control sample. Another study [23] showed an improvement in TPC, together with a noticeable increase in antioxidant activity to 34.191% after 28 days of fermentation. Meanwhile, the antioxidant activity was only 17.005% in control sample. Further, it was found that an increase in TPC was also followed by antioxidant activity increase of 13.6 times higher than control sample, and the antioxidant activity recorded was extremely strong [6]. Sitanggang et al. [27] also found a considerable improvement in antioxidant activity in lentils fermented for 24 h using *Aspergillus oryzae* to 6.81 μmol TE/g, compared to 5.82 μmol TE/g in a control sample. The solid-state fermentation, utilizing okara, performed by Sitanggang et al. [27] indicated 5.5 times higher antioxidant activity as well. The antioxidant activity observation [17] also showed that the antioxidant activity was 15.4 mg AAE/g dwb after 4 days of fermentation. It was 2.1 times higher than the control sample which was 7.3 mg AAE/g dwb.

In this study, different concentrations of $(NH_4)_2SO_4$ were necessary to obtain maximum TPC (0.680% w/v), and maximum TAC (0.087% w/v). In research conducted by Schmidt and Furlong [28], ammonium sulfate in the fermentation media affected biomass production because ammonium sulfate functioned as a source of nitrogen in the fermentation media. With increasing amount of biomass fermented by microorganisms, phenolic released into the extract also increased. However, there has not been literature

found about correlation between concentration of $(NH_4)_2SO_4$ and antioxidant activity in a fermentation process. In a study completed by Porto et al. [29] about tomatoes that were added with a source of nitrogen into their growing media, it was known that increasing the ammonium sulfate concentration as the source of nitrogen could enhance the TPC in the tomatoes. In other words, to gain higher TAC, lower concentration of ammonium sulfate was needed. It was argued that molecules of phenolic compounds were crucial to inhibit free radicals [30], and it was concluded that TPC and TAC was highly correlated [30, 31].

Enhancement of the total phenolic content (289.69 mg GAE/g compared to 86.06 mg GAE/g without fermentation) and total antioxidant capacity (50 ppm compared to 600.91 ppm without fermentation) of sugar palm fruit resulted in the end of 12-day solid state fermentation using *Aspergillus oryzae,* prove that the method can be used to improve total phenolic content and total antioxidant capacity. Based on these results also show that sugar palm fruit has potential to be a source of antioxidant compounds.

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Authors' Contributions. The research was designed, the data was analysed and the manuscript was written by MMM; the research was designed and the manuscript was reviewed by RFK; the research was designed and the process was supervised by AM; the research process was conducted and the data were collected by HNH and TMPL. All authors read and approved the final manuscript.

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