Original Article

Antioxidant activity of endophytic bacteria origin from endocarp of cocoa pod husk

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Abstract

Cocoa (*Theobroma cacao* L.) is one of the leading commodities in Indonesia. Cocoa pod husk is part of the mesocarp or part of the cacao pod husk that wraps the cocoa beans. Endophytic bacteria are microbes that live in host plant tissues and do not interfere with host plant growth. This study aimed to determine characteristics of endophytic bacterial isolates contained in endocarp cocoa pod husk and to determine the antioxidant power of endophytic bacterial isolates contained in cocoa pod husk. Isolation of endophytic bacteria from the inner pod of cocoa which be expected to produce antioxidants has been carried out. Isolation of bacteria using surface sterilization method. Antioxidant test using DPPH method (1,1-diphenyl-2-picrylhydrazyl) with UV-Vis spectrophotometer at 600 nm. Two isolates out of four endophytic bacteria were obtained from the results of the preliminary test of phenolic tests, namely isolates of bacteria KK2 and KK3 which had different colony morphology. The results of the secondary metabolite test showed that the bacterial isolates KK2 and KK3 contained flavonoids and phenolics. Based on the test results, the value of IC_{50} of bacterial isolate KK2 was 2104.58 ppm and KK3 bacterial isolate was 1123.04 ppm. The highest antioxidant activity was possessed by bacterial isolate KK3 and was classified as a very weak antioxidant.

Keywords: antioxidant, cocoa pod husk, DPPH, endophytic bacteria
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Introduction

Cocoa (*Theobroma cacao* L.) is one of the main ingredients in Indonesia. Badan Pusat Statistik (BPS) reports that in 2020 cocoa production reached 716.6 thousand tons on smallholder plantations, 3.08 thousand tons on private plantations and 0.98 thousand tons on large state plantations. If the total cocoa production in Indonesia in 2020 will reach 720.66 thousand tons (BPS, 2020).

Cocoa husk is the main residue in the processing of cocoa beans and constitutes 70% of the total fruit. According to Campos-Vega *et al*. (2018), from the processing of cocoa beans, waste by-products are obtained cocoa pod shells reach 76% w/w or seen from the 2020 cocoa pod production data reached 720.66 thousand tons, the by-product in the form of cocoa shell was 547.7 thousand tons/year.

Cocoa pod husk has an antioxidant activity in IC_{50} of 15.46 ppm (Ulfa *et al*., 2019). Cocoa pod husk contains chemical components lignin, polyphenols and theobromine. Polyphenols are compounds that play a role in giving color to plants and can act as antioxidants that can delay, slow down, and prevent the process of lipid oxidation by free radicals that cause various diseases. Bioactive compounds such as antioxidants in cocoa pod skin can be obtained through the extraction process (Giskha *et al*., 2022). Endocarp cocoa pod husk is the part of the pod that is in contact with the cocoa bean, which is known to have high antioxidant activity.

Antioxidants work by overcoming the damage caused by free radicals on human skin. Free radicals are an important factor in the aging process and damage to skin tissue. The deleterious effects of excess free radicals, or oxidative stress, have been reported to eventually lead to cell death. The overproduction of reactive oxygen and nitrogen species has been implicated in the development of various chronic and degenerative diseases. It is known that the deleterious effects of free radicals can be controlled by specific antioxidant systems (Martemucci, *et al*., 2022). Antioxidants inhibit the oxidation process, even at relatively low concentrations, and can protect cells against free radical damage by delaying or preventing the oxidation of proteins, carbohydrates, lipid, and DNA.

These antioxidants may also be produced by endophytic microorganisms that live on cocoa which have high antioxidant compound. Endophytic microbes are microorganisms that are present in the host plant tissue, but do not interfere with the growth of the host plant. Endophytes generate the same signal pathway as their hosts by during gene mutation or information exchange and then produce secondary metabolites similar to their hosts (Li*, et al*., 2022). Endophytic bacteria derived from plants are capable of creating the same secondary metabolites from native plants. Each higher plant can contain several endophytic microbes that produce secondary metabolites as a result of coevolution or genetic transfer (genetic recombination) from the host plant to the endophytic microbes. Studies based on metabolomics have also shown that endophytes are repositories of bioactive metabolites that can produce many active products with pharmacological effects, such as antimicrobial, antitumor, antibiotic, antioxidant, and immune agents (Gupta, *et al*., 2020). The active products of endophytes can solve the shortage of natural resources and provide new age ideas for the development and preparation of new drugs. At present, some endophytic bacteria and their active products have been successfully used in commercial production and obtained great benefits in the preparation of new drugs and agricultural protection (Mishra *et al*., 2018). Isolation of endophytic bacteria on cocoa pod husk which has potential as an antioxidant has not been widely explored. Isolation of endophytes from cocoa pod husk can be used as an

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antioxidant material so as to reduce cocoa pod husk waste.

Methods

This research was conducted at the Microbiology Laboratory of the Institut Teknologi Sumatera. This research was conducted from March to August 2022. This research was conducted to isolate endophytic bacteria from the endocarp of cacao pod husk and determine their potential as antioxidants. Endophytic bacterial isolates were characterized based on macroscopic and microscopic characteristics. The isolates obtained were then separated based on their morphology and tested for phenolic and antioxidant properties.

Endophytic bacteria isolation

Cocoa pod husk were taken from cocoa plantations in East Lampung Regency, Lampung, with the criteria of cocoa that was ripe and not infected by pathogens. Cocoa pod husk then sterilized by immersion method with 70% alcohol solution, for five minutes with 5.25% NaOCI solution, rinse three times repetition with sterile distilled water (Putri and Herdyastuti, 2021). The cocoa husk is separated for the endocarp. Steriled endocarp cutted into sizes 1x1 cm and then crushed using a mortar to form a mush, then grown on nutrient broth (NB) media and incubated for 24 hours at room temperature. The dilutions are made up to 10^{-6}. At a dilution of 10^{-4} - 10^{-6}, sample were inoculated into nutrient agar medium using the spread plate method and incubated for four days at room temperature and carried out 2 repetitions. The mixed culture was isolated into nutrient agar (NA) medium at room temperature for twenty four hours, then pure colonies were obtained. Bacterial colonies growing from endocarp cocoa pod husk were observed for their morphology, then separated based on their morphological differences.

Fenolic test

Methanol solution 0.5 ml, 1 ml of gallic acid solution, and 1 ml of sample solution were put into 3 test tubes. Then given 5 ml of Folin-Ciocalteu reagent previously purified using distilled water at a ratio of 1:10. After mixing evenly, 4.0 ml of sodium carbonate solution was added. Wait up to 10 minutes then observe the color change in the solution. The results for this test if there is a change in color to blue are categorized as positive for phenolic content (Widodo and Soegihardjo, 2012).

Bacterial characterization

Observation of bacterial morphology was carried out by gram staining method. The bacterial isolates from the liquid media were streaked onto the glass slide which had previously been sprayed with 70% alcohol, passed over the bunsen to dry, then dripped with crystal violet dye and waited for 30 seconds. Rinse and drip Lugol's iodine solution for 30 seconds then rinse again. After that, drop a drop of acetone alcohol, wait until it absorbs and drop safranin solution as a comparison dye for 30 seconds. Then rinse and wait until dry before being observed under a microscope. Observations were made using a microscope at 1000x magnification to show the shape and color of the bacterial cell wall.

Bacterial growth curve

One ose of bacterial isolates was inoculated into 10 mL of nutrient broth media and incubated at room temperature for 24 hours which was used as the first starter. Two milliliters of starter was planted in 18 mL of NB medium and incubated in a shaker at room temperature for 24 hours as a second starter. A total of 20 mL of the second starter was planted in 180 mL of NB medium. The absorbance of liquid media was measured at 0 hour and every 1 hour thereafter by taking 4 mL of bacterial culture. The bacterial culture was centrifuged at 6000 rpm at a low temperature of 4°C for 10 minutes. The residue was added with 5 mL of distilled water and vortexed until homogeneous. Turbidity is determined by measuring the absorbance value at a wavelength of 600 nm using a UV-Vis spectrophotometer. Observations were made for 24 hours to produce OD value data for the y-axis and observation time for the x-axis so that the growth curve can be determined. The growth curve shows the growth phase of the bacteria. The optimum time for the production of secondary metabolites from bacteria is at the end of the stationary phase towards the death phase (Rahman, 2016).

Production of secondary metabolites of endophytic bacteria

A total of 2 mL of regenerated culture was inoculated into 200 mL of NB medium and incubated in a shaker incubator at room temperature. According to the results of the growth curve obtained, the culture is harvested when the stationary phase approaches the death phase. The next step was centrifugation at 6000 rpm, 4°C for 10 minutes. Furthermore, the supernatant was purified. The supernatant was then immersed in a 96% ethanol solution for seventy-two hours at a ratio of 1:3 and then evaporated at 56°C at a speed of 60 rpm and then heated in an oven at 30°C for 72 hours and a concentrated extract was obtained.

Antioxidant activity test

Measurement of antioxidant activity was determined based on the DPPH method. 2 mg was dissolved with the compound 1,1-diphenyl-2-phcrylhydrazyl into 50 mL methanol solution. Then 4 mL of 1,1-diphenyl-2phcrylhydrazyl solution was taken and added with 1 mL of metabolite extract in various concentrations, namely 500 ppm, 250 ppm, 125 ppm, 62.5 ppm, 31.25 ppm. Then the mother liquor was allowed to stand for 30 minutes in a dark room. Analysis used a UV-Vis spectrophotometer which was carried out at the highest wavelength of 600 nm. The IC_{50} value is the price obtained by entering 50% for the y value in each equation obtained for each test sample. In particular, compounds are categorized as a substance has antioxidant properties if the IC_{50} value is less than 200 ppm. If the IC_{50} value obtained is in the

range of >200 ppm, then the substance is less active but still has potential as an antioxidant (Molyneux, 2004).

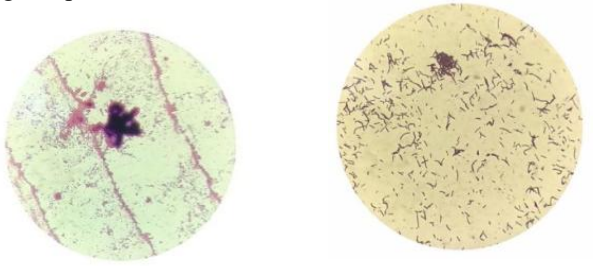
Results

The isolates obtained during purification were 4 bacterial isolates, namely KK1, KK2, KK3 and KK4 (Table 1). Based on the morphological observations of endophytic bacterial isolates, isolates KK1, KK2 and KK4 were white, while KK3 was yellow. The shape of isolates KK2, KK3 and KK4 has an irregular shape, while KK1 has a circular shape. The edge on isolate KK1 is the entire KK3 curled, while isolates KK2 and KK4 have an undulate edge. All isolates have a flat elevation.

Table 1. Morphological characters of bacterial isolates from cocoa pod husk endocarp

Isolat	Colour	Shape	Edge	Elevation
KK1	White	Circular	Entire	Flat
KK2	White	Irregular	Undulate	Flat
KK3	Yellow	Irregular	Curled	Flat
KK4	White	Irregular	Undulate	Flat

The phenolic test was carried out on all isolates to determine the presence or absence of phenolic compounds produced by endophytic bacterial isolates from cocoa pod husk. The results of the phenolic test all isolates tested positive for containing phenolic compounds, which indicated a change in color to blue. There is a change in the blue color which is increasingly concentrated so that the higher the phenolic content contained in the endophytic culture. Two isolates with a high level of blue concentration are KK2 and KK3. In screening of phenolic compounds, methanol solution was assumed to be a negative control, while gallic acid was used as a positive comparison control. Isolate with the highest phenolic content was used for the next test.



KK2 **KK3**

Figure 1. Gram stain results on endophytic bacterial isolates

In Gram staining bacteria isolates KK2 and KK3 were used because these isolates were positive for phenolic content and had a deep blue color. The results of the gram-negative bacteria showed that the bacterial isolates KK2 and KK3 were gram-negative bacteria marked by the presence of a red color on the bacterial cell wall after being treated with a staining reagent (Figure 1). The results of gram staining showed that isolates KK2 and KK3 had a coccus shape.

Based on the growth curve in Figure 2, the KK2 stationary phase began at 19 hours and KK3 began at 15 hours. The results of the growth curves obtained showed that the endophytic bacteria KK2 and KK3 entered the stationary phase at 21 hours. From the OD data, secondary metabolite harvesting of endophytic bacterial isolates was carried out at 21 hours.

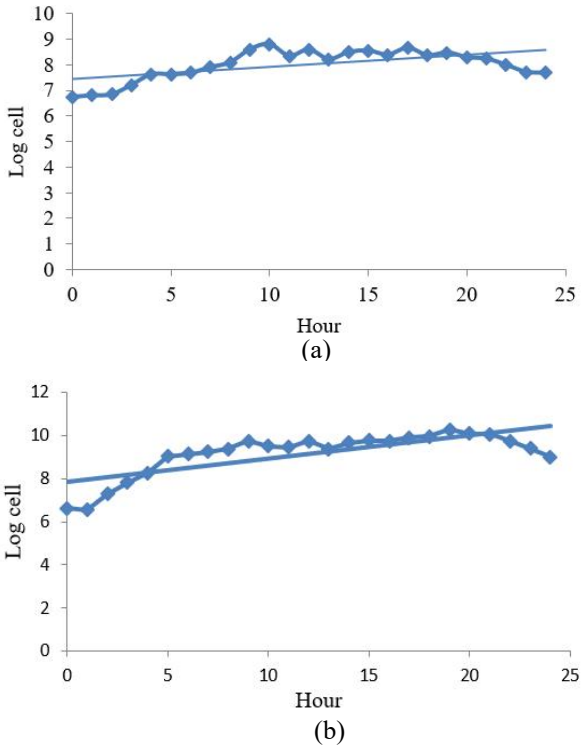


Figure 2. Bacteria growth chart (a) KK2 isolate (b) KK3 isolate

Based on the results of the antioxidant activity test, it was found that isolates KK2 and KK3 had weak antioxidant levels (Table 2). A compound is categorized as a very strong antioxidant if the IC_{50} value is less than 50 ppm, and the lower the IC_{50} value, the higher the antioxidant activity. IC_{50} is a value that results in a concentration of absorbing 50% of the free radical DPPH.

Table 2. Test results of antioxidant activity of endophytic bacterial isolates

Isolate	IC_{50} (ppm)
KK2	2104,58
KK3	1123,04

Discussion

Prior to the endophytic isolation process, the endocarp sample of the cocoa pod husk was surface sterilized first. Sterilization on the skin surface which aims to remove non-endophytic microorganisms and also kill thick-walled spores, as well as some harmful fungi. Four endophytic bacterial isolates obtained from the endocarp of the cocoa pod husk positively contained phenolic compounds based on qualitative tests.

The phenolic test establishes the Folin-Ciocalteau principle based on redox reactions. The principle of measuring phenolic levels uses the Folin-Ciocalteau reaction, namely the occurrence of complex compounds with a blue color (Widodo and Soegihardjo, 2012). The formation of blue color change is due to the phenolic compounds binding to the Folin-Ciocalteau solution in

an alkaline environment, resulting in the breakdown of protons to phenolic compounds (Camarena, *et al*., 2018).

In the phenolic test results, all positive isolates contained phenolic with a change in color to blue, but there were two isolates that were dark blue, namely isolates with the codes KK2 and KK3. It is assumed that isolates that produce deep blue are due to the large amounts of phenolic content produced by isolates KK2 and KK3. Isolates that are phenolic positive are most likely to have antioxidant activity. The higher the phenol content, the stronger the antioxidant activity. Phenolic compounds are important plant constituents with redox properties responsible for antioxidant activity. The hydroxyl groups in plant extracts are responsible for facilitating free radical scavenging (Aryal, *et al.*, 2019). Phenolic compounds are good electron donors because their hydroxyl groups can directly contribute to antioxidant action. Furthermore, some of them stimulate the synthesis of endogenous antioxidant molecules in the cell. Phenolic compounds can exhibit free radical inhibition, peroxide decomposition, metal inactivation or oxygen scavenging in biological systems and prevent oxidative disease burden (Babbar, *et al*., 2015).

Based on gram staining, isolates KK2 and KK3 were gram negative. Gram-negative bacteria have cell walls that are more complex and also thicker than gram-positive bacteria. Based on Rahman's research (2016), it was found that endophytic bacteria belonged to the gram-positive group with diplobacill cell shapes. In general, endophytic bacteria are very diverse so that different cell shapes are obtained in each plant.

As microbial diversity is huge, the mechanisms of responses and growth rates for different microorganisms under a broad range of conditions are expected to be similarly diverse. The growth rate in a continuous culture does not depend on other factors such as nutrient concentration in the medium. Varying the culture medium concentration will increase or reduce cell abundance in the culture but the growth rate of the cells should remain constant (Gonzales and Aranda, 2023). The growth of a bacterial isolate in the laboratory is represented by a sigmoidal curve composed of differentiated growth phases. The lag phase of growth (initiation of the metabolic machinery for growth), the exponential phase of growth (exponential increase in cell abundance and maximum growth rate), the stationary phase of growth (cells adapt to changing conditions due to scarcity of nutrients), and the decline (or death) phase (decrease in cell number) (Joegersen and Wichern, 2018).

Both endophytic bacteria KK2 and KK3 entered the stationary phase at 21 hours. This is a period that generally is represented by zero net growth. Some cells might be dying while others might take advantage of the released nutrients as result of cell lysis to keep growing at a limited rate (Escudero, *et al*., 2018). As the stationary phase of growth progresses, conditions become increasingly adverse and so growth becomes more limited over time. Cell death might increase, leading to the decline or decay (death) phase of growth. The ultimate goal of changes in the microbial population is to maximize survival (Reyes, *et al*., 2020).

Bacterial isolates KK2 and KK3 entered the death phase at 21 hours. The death phase is characterized by a continuous decrease in OD. In the death phase, more cells die than new cells due to a lack of growth factors such as vitamins and minerals (Zhang, *et al*., 2021). Secondary metabolites are produced by bacteria when the bacteria are experiencing a stationary phase nearing death.

Bacterial isolates KK2 and KK3 entered the death phase at 21 hours. The death phase is characterized by a continuous decrease in the absorbance value. In the death phase, there are more dead cells than new cells due to a lack of growth factors such as vitamins and minerals (Susanti, 2021). Secondary metabolites are produced by bacteria when the bacteria are experiencing a stationary phase nearing death. Based on absorbance data, secondary metabolite harvesting of endophytic bacterial isolates was carried out at 21 hours. This phase is where the number of bacterial cells is balanced, in other words the number of bacteria that grow is equivalent to the number of bacteria that die. The occurrence of death in the population occurs due to competition between bacteria to defend themselves. As a result of the limited nutrition in this phase, the bacterial population metabolizes to produce secondary metabolites (Seyedsayamdost, 2019).

The antioxidant IC_{50} value of isolates KK2 and KK3 belong to the very weak category. IC_{50} is a numerical value to determine the concentration value in the extract that can prevent the oxidation process by 50%. The lower the IC_{50} value, the higher the antioxidant activity. If the IC_{50} concentration of an extract is less than 50 ppm, the antioxidant activity is classified as very strong, IC_{50} is classified as strong, 50-100 ppm, moderate 100-150 ppm, and IC_{50} 151-200 ppm is weak. If the value is more than 200 ppm, the antioxidant activity is classified as very weak[(14)]. The antioxidant power of KK3 is higher than that of KK2, as seen from the IC_{50} KK3 < KK2 value. The high value of IC_{50} is assumed because during extraction the sample was still mixed with Nutrient Broth (NB) media. This resulted in the extract sample being dilute, so the IC_{50} value was at a very high concentration.

Conclusion

Based on the results of the study, it can be concluded that four bacterial isolates were obtained from cocoa pod husk, namely isolates KK1, KK2, KK3 and KD4 which were gram negative in the form of coccus. The antioxidant activity (IC_{50} value) of isolate KK2 was 2104.58 ppm and isolate KK3 was 1123.04 ppm and belonged to the very weak category of antioxidant activity.

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