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Cultivation of Antioxidant-Producing Endophytic Fungus in *Rhizophora apiculata* Mangrove Plants

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Abstract: West Sumatra is known as one of the largest areas of mangrove forests in Indonesia, dominated by Rhizophora. Therefore, there will be huge opportunities to discover new types or strains of endophytes and antioxidant compounds, which play an important role in protecting cells caused by free radicals, preventing oxidation, and protecting the human body from ROS. This study aims to determine the optimal pH and temperature for growth and antioxidant activity of endophytic fungal isolates of mangrove plants *Rhizophora apiculata* using descriptive design with DPPH (2,2-diphenyl-1-picrylhydrazyl) method as determination of antioxidant activity and quantitative analysis of IC50 value. The result showed that the optimum pH for antioxidant activity of isolate EUA-111 was 5.0, EUA-112 was 5.5 and EUA-130 was 6.0. In addition, the optimum temperature for all isolates was 31°C. pH and temperature affect the increase in antioxidant production in endophytic fungal isolates of *R. apiculata*.

Keywords: Antioxidant, Endophyte, pH, Temperature, *Rhizophora apiculata*

Introduction

Indonesia is the country with the largest number of mangrove ecosystems in the world, which is around 42,550 km^2. Mangrove plants in Indonesia are spread across Papua, Java, Sumatra, Sulawesi, Kalimantan, Nusa Tenggara, and Maluku (Berawi and Marini, 2018). Most parts of the oil mangrove plant (*R. apiculata*) are used as medicine by coastal communities in Indonesia because it contains useful active ingredients such as alkaloid, flavonoid, triterpenoid, steroid, saponin, and tannin (Chen *et al*., 2022)).

Endophytic fungi live in plant tissue and are not parasitic to their host. (Bogas *et al*., 2024). Endophytic fungi have diverse types, which are generally classified into the Ascomycetes phylum. The genus of endophytic fungi from several plants that have been successfully isolated includes the phylum Ascomycetes. In endophytic fungi, there is an enzymatic process related to the relationship between the host plant and the endophyte which produces several phytochemicals (alkaloids, steroids, terpenoids, coumarin derivatives, quinones, flavonoids, phenols, etc.,) that are characteristic of the corniculatum has the ability of antioxidant activity > IC50 = 19.28 µg/mL (Nurhalimah *et al*., 2021). The results of research from Berawi and Marini (2018) on the stem of *R. apiculata* have antioxidant activity >IC50 = 77.12 µg/mL.

Free radicals are molecules that have one or more unpaired electrons, making them relatively unstable. (Ridlo *et al*., 2017). Free radicals can cause damage to body cell tissues such as lipids, proteins, carbohydrates, and DNA, thus free radicals can cause chronic and acute diseases, cancer, and premature aging. According to Liochev (2013), one effective way to prevent free radicals is by consuming foods that contain antioxidant compounds. Antioxidants are chemical compounds that can donate one or more electrons to free radicals so that free radicals can be suppressed. (Budiono *et al*., 2019).

Antioxidants play an important role in protecting cells from damage caused by free radicals. Antioxidants also play a role in preventing oxidation and can protect the body from Reactive Oxygen Species (ROS) (Omodamiro and Ikekeamm, 2016). One alternative source of natural antioxidants is found in marine plants such as mangrove forests. Anthocyanins are one of the compounds that produce antioxidants. According to research by Nurhalimah *et al*. (2021), mangrove endophytic fungi Aegiceras corniculatum can show antioxidant activity with an IC50 value of 19.28 µg/mL using the DPPH method and

based on the results of research by Rahim *et al*. (2008), *R. apiculata* stem bark has antioxidant activity with a maximum concentration of 30 μg/mL.

Antioxidant testing can be done using the DPPH method (2,2-Diphenyl-1-Picrylhydrazyl). DPPH is an antioxidant testing method with the principle of hydrogen capture reaction from antioxidants by DPPH free radicals which are converted into 2,2-diphenyl-1-picrylhydrazyl) which is then measured in intensity using a spectroscopic photometer at a wavelength of 517 nm. DPPH gives strong absorption at a wavelength of 517 nm with a dark purple color. The capture of free radicals causes electrons to become paired which then results in the elimination of color proportional to the number of electrons taken. Antioxidant activity can be obtained by calculating the amount of reduction in the intensity of the purple color of DPPH which is proportional to the reduction in the concentration of DPPH solution through measurement of the absorbance of the test solution. Activity is expressed in IC50, which means the minimum concentration to inhibit 50% of free radicals. The DPPH method is simple, fast, easy, and sensitive to samples with small concentrations (Karadag *et al*., 2009).

In general, the factors that affect the growth of fungi are substrate, humidity, temperature, acidity (pH), and chemical compounds in the surrounding environment (Bogas *et al*., 2024). In addition, fungal species thrive in warm, sweet, acidic, and aerobic conditions. pH or acidity is a factor that affects the growth of fungi in the growing medium. Low and high pH can cause damage to microbial cells and can result in a decrease in microbial metabolic activity. So microbes have different optimal pH to produce their metabolism. pH that is too acidic or pH that is too basic will damage microbial cells. (Dao *et al*., 2023). Temperature is one of the environmental factors that greatly affects the growth of organisms. Each fungus has different optimal, minimum, and maximum temperatures for its growth. Growth at temperatures below the optimal temperature can reduce the average cell metabolism. Temperatures above the optimum, cause growth to decline and death is possible if the maximum temperature is exceeded (Mustafa *et al*., 2023). Based on this, it is necessary to conduct research related to optimal pH and temperature on the growth and antioxidant activity of mangrove endophytic fungal isolates (*R. apiculata*).

Materials and Methods

Biomass Collection

Weighed Eppendorf in an empty state, then filled with 1 mL of inoculum multiplication every 24 h/day then centrifuged for 15 min at 6000 rpm. The supernatant obtained was discarded and the remaining supernatant was washed using sterile distilled water and then centrifuged again. The washing process was carried out twice, after washing and centrifugation the latter was balanced on an Eppendorf. Eppendorf was then incubated in the oven at 80°C and balanced on Eppendorf tubes every day until the weight was constant. Sampling was done at 24-h intervals. Biomass calculations were measured from the constant weight minus the weight of the empty Eppendorf.

Extraction of Fermentation Products

The mushrooms obtained during the fermentation period were separated from the medium and then baked at 40°C for 3 h. After the mushroom was macerated using methanol in a ratio of 1:10 for 2 days in dark conditions. After maceration, the mushrooms were filtered and the filtrate obtained was evaporated using rotary evaporation until the mushroom extract was obtained.

Maceration uses methanol solvent because of its ability to extract polar and non-polar compounds, methanol also has volatile properties that make it possible to produce clean and concentrated mushroom extracts and methanol can maintain the stability of secondary metabolite compounds extracted from endophytic fungi. After maceration, the mushrooms were filtered and the filter results were evaporated using rotary evaporation until the mushroom extract was obtained.

Antioxidant Tags

Production of 0.05 mm DPPH Solution

DPPH powder (BM 394.32) 0.0001 g was dissolved with 100 mL of methanol so that a DPPH solution of 0.05 ppm was obtained. Determination of the maximum wavelength of DPPH was measured with a spectrophotometer at a wavelength between 501-533 nm; the selected wavelength has the highest absorption (Budiono *et al*., 2019).

Preparation of Test Solution

The primary solution was made by dissolving the extract using methanol to obtain a concentration of 1000 ppm. The primary solution was varied into five different concentrations of 500, 250, 125, 62.5, and 31.25 ppm. The concentration variation considers the half-maximum factor (EC50), it can be determined the lowest and highest concentration limits where the organism or process tested gives significant or optimal results.

Antioxidant Test

A total of 1.5 mL of test solution from each concentration was put into a test tube and 3 mL of 0.05 mM vortex DPPH solution was added until homogeneous and incubated in a dark room for 30 min. Then the absorbance was measured at the wavelength obtained. For positive control, standard antioxidant ascorbic acid was used with the same treatment as the sample. (Hameed *et al*., 2017).

The antioxidant activity of the sample was determined by the magnitude of the DPPH radical absorption inhibition through the calculation of DPPH absorption inhibition (Elfita and Masyita, 2015), which can be calculated with the following formulation inhibition = absorbance of blank - absorbance of sample/absorbance of blank ×100%

IC50 Determination

IC50 was determined by using the sample concentration and the percentage inhibition obtained on the x and y axes, respectively, in a linear regression equation with a y value of 50 and an x value to be achieved as IC50 (Suryanita *et al*., 2019). The DPPH absorbance value data for the control and DPPH after being reacted with the sample solution and compared with several concentrations were used to calculate the percentage of inhibition. The percentage inhibition value is then used for the test solution that can produce free radical silencing of 50 with a linear regression equation:

$$Y = a + bx\, y = 50$$

x = concentration of test solution a = Rate b = constant (Hasanah *et al*., 2017).

For each sample, indicate the y value of 50 and the × value obtained from IC50. The IC50 value indicates the amount of sample concentration (extraction and ascorbic acid control) required to reduce DPPH free radicals by 50%. (Flieger *et al*., 2021).

Effect of pH on the Production of Endophytic Antioxidant Isolates

Prepare PDB media with different pH variations by adding 1 N NaOH and 1 N HCl. The pH variations used are 5.0; 5.5; 6.0; 6.5; and 7.0. The fermentation process was then carried out with a 100 rpm shaker at 30°C for the optimal time obtained in the mold growth curve test. The samples were then extracted and tested for antioxidant activity. Fungi are able to grow on a laboratory scale in a fairly wide pH range, which is between 4.5 and 8.0 with an optimum pH between 5.5 and 7.5 depending on the type of fungus. pH too acidic or pH too basic will damage microbial cells (Dao *et al*., 2023).

Effect of Temperature on Antioxidant Production of Endophytic Isolates

The fermentation process was carried out using PDB medium and fungal isolates with different temperature variations, namely 27; 29; 31; 33, and 35°C with the optimal pH level obtained in the previous pH effect test. The fermentation process was carried out with a 100 rpm shaker for the optimal time obtained from the mold growth curve test. The fermentation results were extracted and then tested for antioxidant activity. In general, fungi grow optimally at a temperature of 250-400 C. Fungal growth at temperatures below the optimum temperature can reduce the average cell metabolism. Temperatures above the optimum, because growth decreases and death is possible if it exceeds its maximum temperature (Mustafa *et al*., 2023).

Results and Discussion

Growth Curve and Antioxidant Activity Test Curve

The biomass growth curves and antioxidant activity test curves of fungal isolates EUA-1111, EUA-112, and EUA130 are shown in Figs. 1-3.

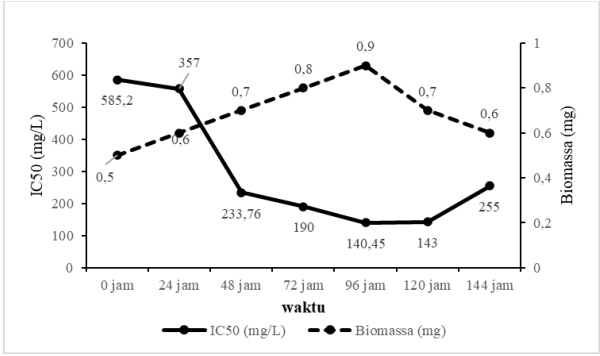

Fig. 1: Biomass and antioxidant activity curves of isolate EUA-111

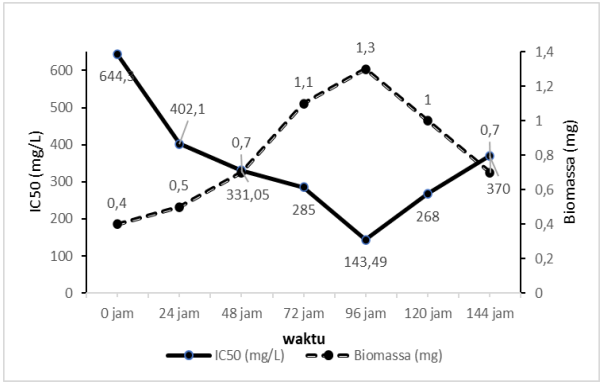

Fig. 2: Biomass and antioxidant activity curves of isolate EUA-112

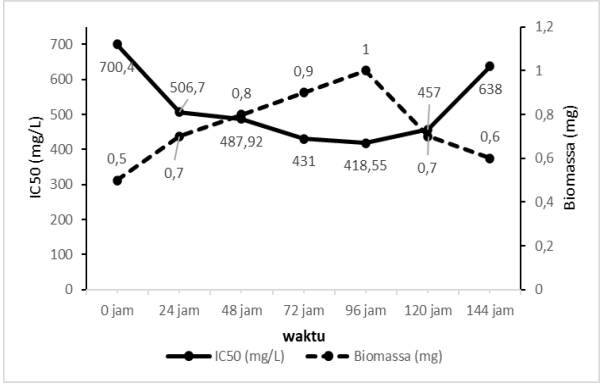

Fig. 3: Biomass and antioxidant activity curves of isolate EUA-130

In determining the optimal time of growth and antioxidant activity of endophytic fungi, their biomass value and antioxidant activity can be used. It can be seen from Figs. 1-3 that the growth of fungal isolates 111, 112, and EUA-130 with the highest biomass value was observed at the 96th h of incubation on the order of 0.9, 1.3, and 1 mg, respectively. Antioxidant activity testing showed that a 96-h incubation period produced good antioxidants. In isolate EUA-111, after incubation for 96 h, antioxidant activity was detected with an IC50 value of 140.45 mg/L. In isolate EUA-112, after incubation for 96 h, the IC50 value of antioxidant activity was 143.39 mg/L. In isolate EUA-130, after incubation for 96 h, the IC50 value of antioxidant activity was 418.55 mg/L. According to Zuraida *et al*. (2017), the ratio of antioxidant activity is inversely proportional to the IC50 value, which means the stronger the antioxidant activity, the smaller the IC50 value obtained.

Based on the results of antioxidant activity, the IC50 value obtained for each isolate in the 96-h incubation period is the smallest compared to other incubation periods. According to Mustafa *et al*. (2023), every microorganism has a growth curve, as well as fungi. The growth curve is obtained from the calculation of fungal cell mass or fungal media hardness at a certain time. In the study, the growth curve was obtained by calculating the fungal biomass every 24 h. Mustafa *et al*. (2023) also noted that the growth curve has several phases, including first, the lag phase is a phase where the fungus adapts to the environment, forming enzymes to break down substrates; second, the acceleration phase is the initial phase where cells divide and increase exponentially; third, the exponential phase is a phase of multiplying cells in very large numbers and increasing cell activity. At the beginning of this phase, the fungus will produce enzymes. Fourth, the stationary phase is a phase in which the number of cells increases and the number of cells that die is relatively balanced. This phase produces many secondary metabolite compounds produced by fungi.

Based on the results obtained, the optimal pH for the growth of endophytic fungal isolates fifth, the death phase is a phase in which the number of dead or inactive cells is greater than the number of living cells. At this stage, the fungus begins to experience death. Based on the biomass and antioxidant activity curves of the endophytic fungal isolates, it is known that the isolates have optimal growth and antioxidant activity during the 96-h incubation period. Secondary metabolites are mostly produced by fungi at the end of the exponential growth phase. In this phase, the growth of fungi is very high while the remaining stock of nutrients in the environment may be insufficient for its survivability, hence the fungi produce secondary metabolites that can be harvested at the end of the exponential phase or the beginning of the stationary growth phase, where the growth column has decreased. According to Mustafa *et al*. (2023), growth curves provide information related to environmental factors that affect fungal growth, such as the optimal, maximum, and minimum temperatures of the fungus.

Determination of Optimal pH for Antioxidant Activity of Endophytic Fungus

The method used to determine antioxidant activity in this study is the DPPH method (2,2-diphenyl-1picrylhydrazyl). The DPPH method is an antioxidant activity test using free radicals that are stable at room temperature and will produce a colorless or purple solution when dissolved in methanol. Antioxidant activity is expressed in IC50, which means the minimum concentration to inhibit 50% of free radicals (Umboro *et al*., 2020)

From the research that has been done, the IC50 value is obtained to determine the optimal pH of endophytic fungal growth in antioxidant production in Table 1.

Based on the results obtained, the optimal pH for the growth of endophytic fungal isolates depends on different antioxidant activities. In isolate EUA-111, the antioxidant activity of fungal endophytes was optimal at pH 5, with an IC50 value of 15.54 mg/L. In isolate EUA-112, the antioxidant activity of endophytic fungi was optimum at pH 5.5 with an IC50 value of 12.10 mg/L. In isolate EUA130, the antioxidant activity of the fungus reached.

The optimum pH was 6.0 with an IC50 value of 47.28 mg/L. The Inhibitor Concentration 50 (IC50) is the effective concentration of substances in the sample to inhibit 50% absorption of DPPH. The higher the IC50 value, the lower the antioxidant activity of the sample.

The growth and production of secondary metabolite compounds of microorganisms are influenced by pH conditions because pH can stimulate the activity of several enzymes that catalyze metabolic reactions. Changes in pH are very important for various processes that occur in microorganism cells (Guimarães *et al*., 2004). pH value of the medium affects the permeability of membranes and cell walls. This will affect the process of absorption and release of ions in the growth medium which will affect the growth of fungi.

Table 1: IC50 value of antioxidant activity based on the pH

Isolated Code	IC50 pH				
	5.00	5.50	6.00	6.50	7.00
EUA-111	15.54	133.84	102.45	158.99	204.23
EUA-112	86.86	12.10	48.13	72.06	122.57
EUA-130	333.41	174.54	47.28	107.87	489.68

The pH condition of the medium shows an optimal pH of 5-7 to produce secondary metabolites such as antioxidants (Masudur Rahman Gazi *et al*., 2004) and other bioactive substances. (Papagianni, 2004; Salah *et al*., 2014). Studies have shown that EUA-111 has optimal antioxidant activity at pH 5 of 15.54 mg/L. Previous research also revealed that the mangrove endophytic fungus Aegiceras carnicultum has good antioxidant activity at pH 5.5 of 0.8 mg/L (Nurhalimah *et al*., 2021). From the research that has been done, isolate EUA-112 has very strong antioxidant activity at the same pH of 5.5 with an IC50 value of 12.10 mg/L. Mustafa *et al*. (2023) found that fungi generally like pH below 7.0. And certain types of fungi can grow at a fairly low pH of 4.5-5.5. The study also found that isolate EUA-130 produced very high secondary antioxidant metabolites at pH 6, namely 47.28 mg/L. According to Mustafa *et al*. (2023), substrate pH is very important for fungal growth due to the presence of certain enzymes that can only dissolve a substrate according to its activity at a certain pH.

Based on the results of Heirina *et al*. (2020), Sonneratia alba mangrove endophytic fungi can grow optimally at 37°C with a pH of 7.0. Fitria and Zulaikha (2019) reported that each microorganism has a different optimal pH for growth. Thus, there are differences in optimal pH between each isolate to produce antioxidant activity. Based on the results obtained, the ability of the fungus to experience a decrease in antioxidant production when it has passed its optimal pH can be caused by the inhibition of fungal growth. The factor that causes inhibition of fungal growth is the accumulation of toxic metabolite compounds (Cadamuro *et al*., 2021)

Determining the Optimum Temperature of Antioxidant Activity of Endophytic Fungus

Based on the optimal pH obtained from the previous pH optimization, temperature optimization is then carried out using the optimal pH that has been achieved. The IC50 value for temperature optimization of fungal growth for antioxidant production is different for each fungus. The IC50 value of the antioxidant activity of the three fungal isolates can be seen in Table 2.

Based on the research conducted, the results show that the ability to produce antioxidants at each temperature and isolation is different. EUA-111 is a very strong antioxidant at 31 and 33°C. However, the lowest IC50 value was obtained at 31°C temperature optimization with a value of 16.59 mg/L. The isolate EUA-112 has antioxidant activity with a very high category obtained at 31°C temperature optimization of 47.16 mg/L. In isolate EUA-130, the strongest antioxidant category was obtained at 31°C temperature optimization with an IC50 value of 64.80 mg/L. If the IC50 value is >500 mg/L, then the sample has no antioxidant activity (Jun *et al*., 2003).

Table 2: IC50 values of antioxidant activity at various temperatures for each isolate

	27	29	31	33	35
	213.14	148.72	16.59	25.22	64.13
EUA-112	625.24	108.68	47.16	440.31	499.62
EUA-130	753.54	120.70	64.80	116.28	97504

From this temperature optimization, it was found that each fungal isolate achieved optimal antioxidant production at the same temperature of 31°C with different IC50 values. According to Bhattacharyya and Jha (2011), the optimal temperature for antioxidant activity is between 25 and 30°C. Different temperatures can affect microbial growth in the media. It is assumed that low temperatures will stop the metabolic activity of fungi and high temperatures can kill fungal cells. (Rebbapragada and Kalyanaraman, 2016). At low temperatures, fungi can stop their metabolic activities because, under stress conditions, temperature will result in damage to membrane stability and fluidity, which will determine the sensitivity or tolerance of cells to stress conditions. This will affect many membrane physiological processes and can affect the cessation of metabolic activity (Abu Bakar *et al*., 2020). High temperatures can inhibit the expression of genes involved in fructose, galactose, and glucose metabolism that play a role in carbohydrate metabolism for fungi (Nehls *et al*., 2001). Endophytic fungi are classified as mesophilic microorganisms. Mesophilic microorganisms are a group of microorganisms that can survive in the temperature range of 25-40°C and optimum at 25-37°C (Black and Hawk, 2005).

The difference in IC50 values in isolates indicates that antioxidant activity is closely related to the fungal strain, way of life (substrate grown in nature), and nutrients in the culture medium. Fungal strains are related to the genes they have to produce active ingredients that are antioxidant (Hameed *et al*., 2017), while the nutrients contained in the culture medium can support the formation of secondary metabolites. (Chen *et al*., 2022).

Antioxidant Activity of R. apiculata Endophytic Fungus Isolated Before and After Optimization

The antioxidant activity of endophytic fungal isolates before optimization and after optimization resulted in different IC50 values. The IC50 values of antioxidant activity of endophytic fungal isolates before optimization and after optimization are shown in Table 3.

Changes in the antioxidant activity of *R. apiculata* endophytic fungi can be seen before and after optimization. The isolate EUA-111 obtained an IC50 value of 140.45 mg/L before optimization with a moderate antioxidant activity category. However, after optimization, the antioxidant activity category of isolate

EUA-111 increased very high with the IC50 value being smaller at 47.16 mg/L. Increased antioxidant production also occurred in isolate EUA-112 with an IC50 value before optimization of 144 mg/L in the moderate antioxidant activity category and after optimization, the IC50 of isolate EUA-112 decreased to 47.16 mg/L which means the antioxidant activity category of the isolate increased to very strong. In the EUA-130 isolate, there was also an increase in antioxidant activity with IC50 value before optimization of 419 mg/L with the weak antioxidant activity category, and antioxidant activity increased after optimization to the strong antioxidant category with a value of 64.80 mg/L. Endophytic fungus Trichoderma sp. has antioxidant activity in mangrove meat. *Cytospora rhizophora* and *Seiridium ceratosporum* isolated from *R. sylosa* had very strong antioxidant activity (Zhou *et al*., 2018).

The antioxidant activity value of *R. apiculata* can be compared with the standard antioxidant vitamin C in Table 4.

Vitamin C has high antioxidant activity because the compound is pure without any other compounds. This is different from endophytic fungi which are still mixed with other compounds but have lower antioxidant activity than Vitamin C. Vitamin C is a substance that is often used to compare antioxidant activity. Because of its ability to react with free radical compounds in vitro, vitamin C is often used as an antioxidant (Febriyanto *et al*., 2022).

Vitamin C is also able to capture free radicals with or without the help of enzyme catalysts. It reacts faster than other liquid components with reactive oxygen compounds. Based on the optimization performed and the results obtained, it can be seen that temperature and pH affect the antioxidant activity very significantly of the endophytic fungus *R. apiculate*. Hewage *et al*. (2014) found that one of the simplest and widely used strategies to improve biological activity is to modify culture conditions, as it should produce different metabolites in response to the environment. (Pan *et al*., 2019).

Table 3: Antioxidant activity of *R. apiculata* endophytic

	Before the optimization		After the optimization	
Isolate code	IC50 (mg/L)	Category antioxidants	IC50 (mg/L)	Category antioxidants
EUA-111	140.45	Medium	16.59	Very strong
EUA-112	143.49	Medium	47.16	Very strong
EUA-130	418.55	Weakly	64.80	Strong

Table 4: The antioxidant value of vitamin c standard

Concentration (ppm)	% inhibition	IC50 (mg/L)	Category antioxidant
1000	74.848		
500	73.030		
250	72.121		
125	61.212	11.97	Very strong
62.5	59.091		
31.25	56.364		

Conclusion

The conclusion of this study shows that the antioxidant activity of isolate EUA-111 is optimal at pH 5.0, isolate EUA-112 is optimal at pH 5.5, while isolate EUA-130 is optimal at pH 6.0 with the optimal temperature of all isolates is 31°C. pH and temperature affect the increase in antioxidant production in R. apiculata endophytic fungal isolates.

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Author's Contributions

Anthoni Agustien: Developed the initial idea, wrote the manuscript, designed the research, and reviewed and approved the manuscript.

Putra Santoso: Data analysis, experiment development.

Siti Zaharani Zalamah: Designing research, methodology, data analysis, and manuscript written.

Denny Bendrianis: Materials and equipment involvement and script written.

Mifthahul Jannah: Materials and equipment involvement and monitoring research.

Yetria Rilda: literature search, data analysis, and experiment development.

Muhamad Hafidz Fadjri: Material and equipment involvement.

Erman Munir: Literature search and data analysis.

Ethics

The authors acknowledge the novelty of this article, affirming that it is an original contribution that has not been previously published. All participating authors have thoroughly reviewed and endorsed the content, ensuring there are no ethical issues.

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