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The potential of pineapple peel waste fermentation as antibacterial and antioxidant agents

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ABSTRACT

The fermentation of pineapple peel waste can yield a product in bioactive compounds, including an eco-enzyme that serves as both an antibacterial and antioxidant agent. By utilizing the fermentation process, the extraction of phenolic compounds is enhanced through the action of degrading enzymes produced by microorganisms. This study aims to explore the potential of fermenting pineapple peel waste from with varying fermentation durations of one month (designated as EE-1) and three months (designated as EE-3) to assess their antibacterial and antioxidant properties. The method research is purely experimental methodology with several testing phases: the preparation of the eco-enzyme, a disc diffusion test for antibacterial activity, the determination of the minimum inhibitory concentration (MIC) using the resazurin microtiter assay (REMA), and the assessment of antioxidant activity through the DPPH test. The result showed that the eco-enzyme fermentation has a dark brown color for EE-1 and a light brown color for EE-3, with a pH range of 3 to 3.2 and a sour pineapple aroma. EE-3 exhibited the highest antibacterial and antioxidant activity. It demonstrated strong inhibition against *Propionibacterium acnes* at concentrations of $10^0$and 10^2, with MIC values of 3.13 mg/mL for *Staphylococcus aureus*, 12.5 mg/mL for *Staphylococcus epidermidis*, and 6.25 mg/mL for P. acnes. The minimum bactericidal concentration (MBC) was found to be 25 mg/mL for all tested bacteria, while the antioxidant activity of EE-3 was indicated by an IC50 value of 1.95 mg/mL.

Keywords: Antibacterial, antioxidant, eco-enzyme, fermentation, pineapple peel

INTRODUCTION

The amount of pineapple peel waste has been on the rise since 2017, with production levels surpassing 400,000 tons per year (Prasetyo et al., 2023). Pineapple peels generate a significant amount of waste, accounting for 30% to 42% of the total discarded material (Arimba et al., 2019). Waste refers to the byproduct of a process or usage, which can exist in solid, liquid, or gas forms (Hermawan & Madyasti, 2023). Given this information, numerous researchers have explored the use of pineapple peels in beverages, including tea (Sembiring & Sari, 2021), chip and even in health sector as an antibacterial (Husniah & Gunata, 2020; Lestari & Fitri , 2019; Putri et al., 2018). Several recent studies have also employed pineapple peel waste as a primary ingredient in the production of eco-enzymes for disinfection purposes (Gumilar, 2023; Ningsih et al., 2023; Tallei et al., 2023).

The use of pineapple peel waste in extracts is more frequently reported compared to other applications. Methanol extracts from *Ananas comosus* peels exhibit antioxidant activity with an IC50 value of 46.49 µg/mL, along with moderate antibacterial properties (Putri et al., 2018). Other studies have also indicated that the high polyphenol content in pineapple peels contributes to their significant antioxidant

activity (Cerda-Cejudo et al., 2023; Rivera et al., 2023) but research on fermented pineapple peel waste that can be used as an antibacterial and antioxidant is still limiting.

The fermentation of pineapple peel waste can enhance the extraction of phenolic compounds through conjugate hydrolysis, facilitated by degrading enzymes produced by microorganisms (Ortega-Hernández et al., 2023; Srivastava et al., 2019). The fermentation process can optimize the use of the components found in pineapple peels. In this study, researchers concentrated on transforming pineapple peel waste through a fermentation stage, commonly referred to as eco-enzyme production. The fermentation was conducted over one and three months without the introduction of additional microorganisms. Theoretically, the first month of fermentation yields alcohol, the second month produces vinegar or acetic acid, and the third month generates enzymes. This final product is what is known as an eco-enzyme.

In Indonesia, eco-enzymes are primarily utilized for bioremediation of wastewater, as plant fertilizers, pest repellents, and as alternatives to chemical cleaning agents. However, there has been limited exploration into the potential of fermenting pineapple peel waste for use as an antibacterial and antioxidant agent. The presence of compounds in pineapple peels, such as ascorbic acid, flavonoids, and other phenolic substances, suggests that they may possess antibacterial and antioxidant properties.

In context described above, this study aimed to evaluate the potential of fermenting pineapple peel waste (*Ananas comosus*) as an antibacterial and antioxidant agent, with variations in fermentation duration. The antibacterial activity was assessed using the Resazurin Microtiter Assay (REMA) method, while the antioxidant activity was measured through the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical inhibition method, expressed as the IC50 value.

METHOD

Production of Eco-Enzyme (EE) from pineapple peel

The whole pineapple peels were first chopped into small pieces using a blender. The mixture ratio of sugar, pineapple peel, and water was then determined, set at 1:3:10. Specifically, 900 grams of pineapple peel were combined with 300 grams of brown sugar, which was cut into pieces and melted on the stove before being allowed to cool. This sugar solution was then added to a container containing 900 grams of pineapple peel and 3 liters of sterilized aquadest. The container was sealed tightly, and the mixture was left to ferment for one month (designated as EE-1) and three months (designated as EE-3). After the first month of fermentation, the lid was removed, and the fermented liquid was stirred thoroughly. The container was then resealed and allowed to continue fermenting for an additional three months. Samples were collected from both the one-month (EE-1) and three-month (EE-3) fermentation products, with the three-month fermentation being a continuation of the one-month process. This variation in fermentation duration was implemented to identify the optimal antibacterial and antioxidant activity. The acidity level (pH) of the solution was measured on the first day (before fermentation), as well as one month and three months after fermentation.

Evaluation of inhibition capacity of EE solution against pathogenic bacteria in human skin

Evaluation of the inhibitory ability of EE solution against pathogenic bacteria in human skin are *Staphylococcus aureus* (ATCC® 29522), *Staphylococcus epidermidis* (ATCC® 12228) and *Propionibacterium acnes* (ATCC® 6919). All bacteria were recultured on Natrium Agar and Natrium Broth Media and then incubated for 24 hours at 37ºC. The results on Natrium Broth were compared with the MC Farland 0.5.

Inhibition was assessed using the agar

diffusion method on Muller Hinton Agar (MHA). This approach is commonly employed as a preliminary test to determine whether the eco-enzyme (EE) possesses antimicrobial properties. The EE-1 and EE-3 were diluted from 10^0 to 10^4.

The antibacterial test was conducted by pouring 20 ml of Muller Hinton Agar (MHA) into a petri dish and allowing it to solidify. Once the base layer had set, a 50 µl suspension of the bacterial test was applied to the surface of the MHA and spread evenly using an "L spreader." Subsequently, paper discs with a diameter of 6 mm were prepared in a steril empty petri dish, where they were each infused with 50 µl of EE-1 and EE-2 separately, across four dilutions, with three repetitions for each. Additionally, a cylinder containing 50 µl of ciprofloxacin was used as a positive control and sterile distilled water served as a negative control. The petri dishes were then incubated in an incubator at 37°C for 72 hours.

The clear inhibition area was measured using a caliper and then subtracted from the diameter of the well. According to the classification by Davis and Stout (1971), the diameter of the inhibition zone was categorized based on its antibacterial effectiveness. An inhibition zone with a diameter greater than 20 mm is classified as very strong, 10-20 mm as strong, 5-10 mm as moderate, and 5 mm or less as indicating no antibacterial activity.

Antibacterial activity test Minimum Inhibitory Concentration (MIC) using REMA

Minimum inhibitory concentration (MIC) of both EE-1 and EE-3 against *Staphylococcus aureus* (ATCC® 29522), *Staphylococcus epidermidis* (ATCC® 12228) and *Propionibacterium acnes* (ATCC® 6919) were analyzed with REMA. REMA stands for Resazurin Microtiter Assay. In this antibacterial assay system, resazurin serves as an indicator based on the cells' ability to reduce the blue-colored resazurin compound to the pink-colored resorufin compound.

In brief, 50 µl each well of the following solution contains EE 20 µl, MHB 3.3x 30 µL, Resazurin 10 µL and bacterial suspension ($5x10^6$ CFU/mL) 10 µL. For positive control, Ciprofloxacin was used and Aquadest as negative control. EE 1 and 3-month fermentation period, were mixed in a 96-well plate (Biologix) and incubated for 24 hours at 37ºC. All samples were prepared in triplicates.

The minimum inhibitory concentration (MIC) value is determined by observing the color change in each well. A change in the indicator color from blue to pink indicates that the bacteria are still growing. In contrast, if there is no color change in the indicator, it signifies that the sample is capable of inhibiting bacterial activity. The presence of living bacteria is indicated by a color change from blue to pink or clear. Conversely, if the color of the resazurin reagent remains blue, it indicates that the bacterial cells are dead. The lowest concentration of the test sample that results in bacterial cell death is recorded as the minimum inhibitory concentration (MIC).

Antibacterial activity test using Minimum Bactericidal Concentration (MBC).

The minimal bactericidal concentration (MBC) of EE-1 and EE-3 was determined by selecting samples from the wells in the MIC test that showed no visible bacterial growth and culturing them on MHA agar plates. These plates were then incubated for 24 hours at 37ºC. After incubation, the agar plates were examined visually for any signs of bacterial colony growth. All samples were prepared in triplicate.

Evaluation of the antioxidant activity of EE solution

DPPH radical scavenging assay

The chemicals utilized for the antioxidant assay included DPPH (Aldrich, 1898-66-4), gallic acid (Aldrich, 149-91-7), methanol (Merck, 1.06009.25000), DMSO (Merck, 1.02952.1000), and aquadest. The DPPH radical scavenging assay was performed using a UV-Vis

spectrophotometer, following the method previously reported by our team (Putri et al., 2018).

Antioxidant activity of EE was determined by the IC_{50} value. The EE solutions were diluted to 2.62; 1.31; 0.33; and 0.17 mg/mL with methanol for the 1- and 3-month-old fermented EE. As a radical solution, DPPH was dissolved on a concentration 0of $6x10^{-5}$ M with methanol. Gallic acid served as the positive control. The inhibitory activity against DPPH was expressed as a percentage of inhibition, calculated using the following formula:

$$\text{Inhibition (\%)} = [(Ab-As)/Ab] \times 100\%$$

Where Ab is the absorbance of the blanko (methanol) and As means the absorbance of the samples. The IC_{50} value was determined by analysis of the regression equation linier with the formula y = ax + b, where y = 50 and x is the IC_{50} value.

RESULTS AND DISCUSSION

Eco-enzyme from pineapple peel

The physical characteristics of the eco-enzyme fermentation process, including aroma, color, and pH, were analyzed after one and three months of fermentation. The findings are summarized in Table 1.

Table 1. Eco-enzyme observation

Characteristics	EE-1	EE-3
Aromatic	Fresh fruit acid	Acid fruit
Color	Dark Brown	Light brown
pH	3.2	3.0

Table 1 indicates a distinction between the eco-enzyme fermented for one month and that fermented for three months. As the fermentation duration increases, the pH becomes more acidic, and the color of EE-3 becomes progressively darker brown.

The findings of this study are consistent with those of Natasya et al. (2023) and Farma et al. (2021), who reported that the pH of eco-enzymes ranged from 3 to 3.4, and the color of eco-enzymes varied from light brown to dark brown (Farma et al., 2021; Natasya et al., 2023). During fermentation, microorganisms decompose complex organic compounds into simpler molecules, resulting in the production of various metabolites and organic acids. As these compounds accumulate, they can lead to changes in the pH of the solution (Tallei et al., 2023). The decrease in pH observed in EE-1 and EE-3 after fermentation is most likely due to the accumulation of compounds produced during the fermentation process.

The color change in eco-enzyme (EE) occurs due to the fermentation process. A darker color indicates that fermentation can continue, while a lighter color suggests that the process is complete (Viza, 2022).

The aroma of eco-enzyme (EE) is characterized as slightly sweet, sour, and fresh, which is typical of fermentation. This distinct scent results from the esterification process, leading to the formation of carboxylic groups and the production of acetic acid as the final product. Acetic acid helps inhibit the growth of microorganisms (Viza, 2022).

Eco-enzyme is a cost-effective and efficient alternative for waste processing, capable of producing various organic acids, including acetic, lactic, malic, oxalic, and citric acids (Arun & Sivashanmugam, 2015b). The pH of eco-enzyme (EE) ranges from 3 to 3.2 for both EE-1 and EE-2. Its low pH is attributed to the high concentration of organic acids, where an increase in organic acid content results in a further decrease in pH (Natasya et al., 2023).

Antibacterial activity test using paper disc diffusion method

This preliminary test aims to confirm and assess the antibacterial effectiveness of the EE-1 and EE-3 samples.

Table 2. Inhibition zone (mm) of various concentrations of EE-1

Bacterial	Treatment	Mean ± SD	Category
Staphylococcus aureus	10^0	1.6 ± 1.4	Weak
	10^2	1.6 ± 0.6	Weak
	10^4	0.3 ± 0.6	Weak
	Control +	34.8 ± 4.09	Very strong
	Control -	0.0	-

Bacterial	Treatment	Mean ± SD	Category
Staphylococcus epidermidis	10^0	4.6 ± 1.5	Weak
	10^2	1.9 ± 1.6	Weak
	10^4	0.6 ± 0.5	Weak
	Control +	31.3 ± 5.63	Very strong
	Control -	0.0	-
Propioni-bacterium acnes	10^0	6.0 ± 2.0	Moderate
	10^2	1.3 ± 1.3	Weak
	10^4	0	-
	Control +	30.7 ± 4.25	Very strong
	Control -	0.0	-

Table 3. Inhibition zone (mm) of various concentrations of EE-3.

Bacterial	Treatment	Mean ± SD	Category
Staphylococcus aureus	10^0	6.2 ± 5.33	Weak
	10^2	8.3 ± 11.79	Weak
	10^4	3.2 ± 4.48	Weak
	Control +	34.8 ± 4.09	Very strong
	Control -	0.0	-
Staphylococcus epidermidis	10^0	0.0	-
	10^2	18.2 ± 13.42	Strong
	10^4	3.8 ± 5.42	Weak
	Control +	31.3 ± 5.63	Very strong
	Control -	0.0	-
Propionibacterium acnes	10^0	16.8 ± 11.99	Strong
	10^2	17.7 ± 2.32	Strong
	10^4	0.0	-
	Control +	30.7 ± 4.25	Very strong
	Control -	0.0	-

This study demonstrated that EE-1 and EE-3 exhibit antimicrobial activity. Eco-enzyme derived from pineapple peels contains high levels of protease enzymes, making it effective as an antimicrobial agent against both Gram-positive and Gram-negative bacteria (Saleem & Saeed, 2020). The enzyme content in eco-enzyme is derived from both the fermentation process and the enzymes naturally present in the raw materials used for its production (Ginting & Prayitno, 2022).

The antimicrobial activity of concentrated EE exhibited varying inhibition zones. EE-3 demonstrated the strongest inhibition against *P. acnes*, classified as a strong effect at 10^0 and 10^2 dilutions. Additionally, another study found that eco-enzyme effectively eradicates *S. aureus* bacteria, placing it in the very strong category. (Ginting & Prayitno, 2022). Variations in inhibition zones result from differences sample treatment types (EE-1 and EE-3). The concentration of waste enzymes diffused in the wells also varied between the two studies (Neupane & Khadka, 2019).

According to Evstigneeva et al. (2021), variations in bacterial responses across species are influenced by multiple factors, including abiotic conditions, environmental differences, organ completeness (such as locomotory structures), metabolic activity, and adaptation mechanisms. This preliminary test confirms the potential of eco-enzyme to inhibit or eliminate pathogens, supporting further testing to determine its Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC).

Antibacterial activity test Minimum Inhibitory Concentration (MIC) using REMA

The MIC values of EE-1 and EE-3 at different concentrations, following fermentation periods of 3 and 6 months, are presented in Tables 4 and 5.

Table 4. Minimum Inhibitory Concentration (MIC) with REMA of EE-1 using aquadest solvent

Bacterial	MIC (mg/mL)									
	100	50	25	12.5	6.25	3.13	1.56	0.78	0.39	0.195
S. aureus	1	1	2	2	2	2	2	2	2	2
S. epidermidis	1	1	2	2	2	2	2	2	2	2
P. acnes	1	1	2	2	2	2	2	2	2	2

Note: 1 = color change occurs; 2 = no color change occurs

Table 5. Minimum Inhibitory Concentration (MIC) with REMA of EE-3 using aquadest solvent

Bacterial	MIC (mg/mL)									
	100	50	25	12.5	6.25	3.13	1.56	0.78	0.39	0.195
S. aureus	1	1	1	1	1	2	2	2	2	2

Bacterial	MIC (mg/mL)									
	100	50	25	12.5	6.25	3.13	1.56	0.78	0.39	0.195
S. epidermidis	1	1	1	2	2	2	2	2	2	2
P. acnes	1	1	1	1	2	2	2	2	2	2

Note: 1 = color change occurs; 2 = no color change occurs

This observation was done by visualizing the color change directly. Both EE-1 and EE-3 showed growth inhibite the pathogenic bacteria test. This study shows that the antibacterial efficacy of EE is proportional to its concentration. The higher the concentration, the higher the efficacy. This is in accordance with the findings of previous studies conducted by several studies (Ginting & Prayitno, 2022; Hayati, 2023; Mavani et al., 2020).

The microdilution method for determining MIC offers several advantages, including faster, cost-effective, simple, reliable, and highly sensitive results, making it an effective approach for antibacterial testing of natural products (Putri et al., 2024).

Resazurin was used as an indicator to assess microbial growth, functioning as an oxidation-reduction marker. Commonly known as Alamar Blue, this dye is reduced by aerobic and facultative anaerobic microorganisms (Knapp et al., 2018).

EE-1 inhibition of bacterial growth occurred at 25 mg/mL MIC for all tested bacteria. EE-3 have MIC 3.13 mg/mL for *S. aureus*, 12.5mg/mL for *S. epidermidis* and 6.25 mg/mL for *P. acnes*. This explains that the best EE is EE-3 with the lowest MIC value. The lower the MIC value, the better the antibacterial activity of a sample (Roy & Lingampeta, 2014; Saleem & Saeed, 2020). A minimum fermentation period of three months is required for the preparation of eco-fruit enzymes to achieve optimal levels of hydrolytic enzymes and acetic acid (Arun & Sivashanmugam, 2015a). It is suspected that higher levels of hydrolytic enzymes and acetic acid after a longer fermentation period will help improve the antimicrobial effect of eco-fruit enzymes.

Acetic acid is the primary component in fermentation that contributes to the antibacterial properties of eco-enzyme. While its exact concentration in this study was not determined, previous research suggests that acetic acid levels increase with longer fermentation periods (Arun & Sivashanmugam, 2015b). This occurs as complex organic compounds undergo hydrolysis into simpler molecules through anaerobic fermentation, leading to the accumulation of low molecular weight acetic acid. Due to the pH gradient, acetic acid can penetrate bacterial cell membranes, disrupting their metabolic activity (Lund et al., 2014). Higher osmotic pressure in bacterial cells also causes water entry and cellular osmolysis (Halstead et al., 2015).

Antibacterial activity test using Minimum Bactericidal Concentration (MBC)

Table 6. Minimum Bactericidal Concentration (MBC) of EE-1

Bacterial	MIC (mg/mL)	Result
S. aureus	100	0
	50	0
S. epidermidis	100	0
	50	0
P. acnes	100	0
	50	0

Note: 0 = no bacterial colony growth; 1 = there is growth of bacterial colonies

Table 7. Minimum Bactericidal Concentration (MBC) of EE-3

Bacterial	MIC (mg/mL)	Result
S. aureus	100	0
	50	0
	25	0
	12.5	1
	6.25	1
	3.13	1
S. epidermidis	100	0
	50	0
	25	0
	12.5	1
P. acnes	100	0
	50	0

Bacterial	MIC (mg/mL)	Result
	25	0
	12.5	1
	6.25	1

Note: 0 = no bacterial colony growth; 1 = there is growth of bacterial colonies

The MBC value is determined as a follow-up to the MIC results. After obtaining the MIC, the samples are cultured on media and observed for bacterial growth. The MBC is identified as the lowest concentration capable of eliminating bacteria. The data indicate that the MBC value for the three bacterial strains is 50 mg/mL for EE-1 and 25 mg/mL for EE-3..

The variation in MBC results between EE-1 and EE-2 highlights that higher concentrations lead to greater efficacy. EE-1, with a shorter fermentation period, does not allow for the full development of organic acids, enzymes, and other bacterial metabolic byproducts. According to several studies, a fermentation duration of at least 40–60 days is necessary to optimize bioactive compound production for analysis (Mavani et al., 2020; Ningrum et al., 2024; Permatananda et al., 2023).

Antioxidant activity of EE solution

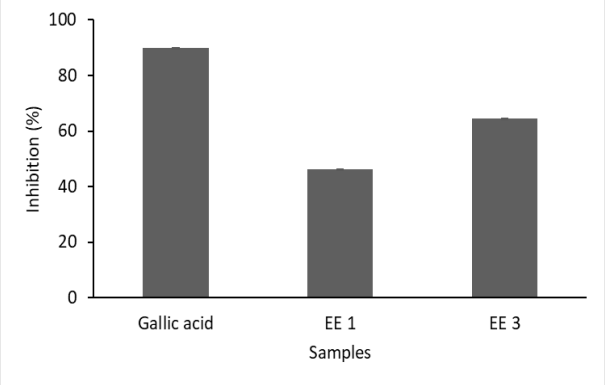

Figure 1. DPPH radical scavenging activity of Eco-Enzyme (EE) with concentration of 2.62 mg/mL

The DPPH radical scavenging assay for Eco-Enzyme (EE) and gallic acid as a standard is presented in Figure 1 and summarized in Table 7. Based on the IC50 data, EE-3 demonstrates strong antioxidant potential against DPPH, with an IC50 value of 1.95 mg/mL. This indicates that EE-3 exhibits higher inhibitory activity against DPPH radicals compared to EE-1. The IC50 value was determined through linear regression analysis, as shown in Figure 2. An IC50 value is only calculated when inhibition exceeds 50%, with lower IC50 values indicating stronger antioxidant activity, meaning less concentration is required to neutralize free radicals.

Table 8. Antioxidant activity of Eco-Enzyme (EE)

No	Samples	DPPH scavenging activity	
		Inhibition (%) ± SD	IC_{50} (mg/mL)
1	EE 1	46.21 ± 0.017	NS
2	EE 3	64.52 ± 0.007	1.95
3	Gallic acid	89.97 ± 0.005	$0.54x10^{-3}$

*values represent the means ± standard deviations for triplicate experiments not studied

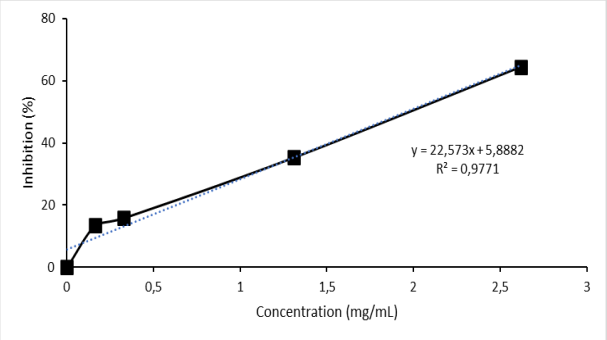

Figure 2. Antioxidant activity of the EE 3

Previously, antioxidant activity of ecoenzyme prepared from combination of papaya, pineapple, and kasturi orange fruits represented to very strong antioxidant activity against DPPH radicals with IC_{50} value of 29.56 ppm for 3 months of fermentation (Tallei et al., 2023). In this study, we specifically focused on eco-enzyme derived from pineapple peel. As a result, the antioxidant activity of EE-3 was lower than that reported in previous studies. According to literature, there is a direct correlation between the fermentation duration of eco-enzyme and its antioxidant activity, meaning that longer fermentation periods enhance antioxidant potential.

CONCLUSION

Fermenting pineapple peel waste for one to three months enhances its antioxidant and antibacterial properties. EE-3 exhibits the highest antibacterial and antioxidant activity. EE-3 has strong inhibition category for *P.acnes* at 10^0 and 10^2*;* MIC value is 3.13 mg/mL for *S. aureus*, 12.5mg/mL for *S. epidermidis* and 6.25 mg/mL for *P. acnes*; MBC for all MIC value; and antioxidant IC_{50} EE-3 = 1.95 mg/mL.

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