Comparison of the Antibacterial Activity of Orange Peel Eco-Enzyme against *Staphylococcus aureus* and *Escherichia coli*

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Abstract

Orange peel contains various bioactive compounds known to possess antibacterial and antioxidant activities, making it a potential raw material for eco-enzyme production. This study aimed to determine the concentration of orange peel eco-enzyme that exhibits the highest antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*, as well as to compare the antibacterial activity of orange peel eco-enzyme against *Staphylococcus aureus* and *Escherichia coli*. Another objective was to evaluate differences in antibacterial activity at different incubation times. This study employed an experimental design, involving antibacterial activity testing of orange peel eco-enzyme using the Kirby–Bauer disk diffusion method. The independent variable was the concentration of orange peel eco-enzyme at 70%, 80%, 90%, and 100%. Other independent variables included bacterial species and incubation times of 18 and 24 hours. The overall statistical analysis showed that the eco-enzyme exhibited significant antibacterial activity against both *Staphylococcus aureus* and *Escherichia coli*, with the optimal concentration being 90%. The Mann–Whitney test indicated no significant difference in antibacterial activity between *Staphylococcus aureus* and *Escherichia coli* ($p > 0.05$). Comparison between incubation periods of 18 and 24 hours also revealed no significant difference in antibacterial activity ($p > 0.05$), indicating that the orange peel eco-enzyme has good stability of antibacterial activity against both test bacteria.

Keywords: orange peel eco-enzyme, antibacterial activity, incubation time, *Staphylococcus aureus, Escherichia coli*

1. INTRODUCTION

Disinfectants are essential components in efforts to prevent and control the spread of pathogenic microorganisms, both in healthcare facilities and in domestic environments. The massive increase in disinfectant use, particularly since the COVID-19 pandemic, has led to a substantial release of active chemical compounds into aquatic environments, soils, and wastewater treatment systems. Various active ingredients in conventional disinfectants, such as benzalkonium chloride, chlorine, phenolic compounds, and hydrogen peroxide, are known to exhibit toxic effects on aquatic organisms and to contribute to long-term ecotoxicological risks. Compounds such as benzalkonium chloride and phenolic substances have even been reported to have high risk quotient values for freshwater organisms, raising serious concerns regarding ecosystem sustainability. These conditions highlight the urgent need for the development of alternative disinfectants that are microbiologically effective yet environmentally friendly (Musee *et al.,* 2023; Ng *et al.,* 2025).

One approach that has recently attracted attention is the utilization of eco-enzymes, which are fermentation liquids produced from organic waste such as fruit and vegetable residues with the addition of sugar and water. The fermentation process generates various bioactive compounds, including organic acids, enzymes, and secondary metabolites that are known to possess antimicrobial activity (Vidalia *et al.,* 2023). In addition to their potential as natural disinfectants, eco-enzymes also offer a sustainable solution for organic waste management (Erlinawati *et al.,* 2025).

Eco-enzymes are fermentation liquids derived from organic materials (such as fruit and vegetable waste) combined with sugar and water under anaerobic conditions for a certain period. Eco-enzymes typically have a pH of approximately 3–4 and contain organic acids (acetic acid, citric acid, and formic acid) produced as microbial metabolites, enzymes such as protease, amylase, and lipase, and volatile compounds such as alcohols, esters, and phenolic compounds generated during fermentation. These compounds play important roles as natural antibacterial agents (Mavani *et al.,* 2020; Galintin *et al.,* 2021; Gumilar *et al.,* 2025). An adequate fermentation period allows microorganisms to optimally produce secondary metabolites (Gu *et al.,* 2021; Febrianti *et al.,* 2024).

Eco-enzymes can be utilized as antifungal, antibacterial, insecticidal, and cleaning agents. Eco-enzymes produced from pineapple, papaya, and orange have demonstrated significant antibacterial activity against *Staphylococcus aureus* and *Escherichia coli,* along with high antioxidant capacity (Vama and Cherekar, 2020; Gayanti *et al.,* 2023; Irianto *et al.,* 2023; Tallei *et al.,* 2023). The antibacterial activity of eco-enzymes is mainly attributed to the combined effects of bioactive fermentation products, including organic acids, enzymes, and secondary metabolites (Permatananda *et al.,* 2023). Eco-enzymes exhibit considerable antimicrobial activity even at various dilution levels, making them promising candidates for environmentally friendly natural disinfectants applicable to sanitation and organic waste management (Vidalia *et al.,* 2023; Lubis *et al.,* 2025). Previous studies have shown that orange peel eco-enzymes possess antibacterial activity against pathogenic bacteria, which is associated with the presence of phenolic compounds, flavonoids, and organic acids produced during fermentation (Erlinawati *et al.,* 2025).

This study aimed to determine the concentration of orange peel eco-enzyme that exhibits the highest antibacterial activity against *Staphylococcus aureus* and *Escherichia coli,* as well as to compare the antibacterial activity of orange peel eco-enzyme against these two bacterial species. Another objective was to evaluate differences in antibacterial activity at different incubation times. The variation in incubation time was applied to assess the stability of the antibacterial efficacy of the eco-enzyme.

2. METHOD

This study employed an experimental design, involving antibacterial activity testing of orange peel waste eco-enzyme using the Kirby–Bauer disk diffusion method against *Staphylococcus aureus* and *Escherichia coli*. The research was conducted at the Microbiology Laboratory of the Medical Laboratory Technology Program, Politeknik Katolik Mangunwijaya. The independent variable was the concentration of orange peel waste eco-enzyme at 70%, 80%, 90%, and 100%, based on a previous study (Irianto *et al.*, 2023); in addition, incubation times of 18 and 24 hours were applied. The dependent variable was the diameter of the inhibition zone around the disks impregnated with the eco-enzyme. Chloramphenicol (30 µg) disks were used as the positive control, while sterile distilled water was used as the negative control.

Research Materials

The equipment used in this study included glass jars, an analytical balance (Ohaus, US), an incubator (Memmert, Germany), a hotplate (Thermo Scientific, US), an autoclave (Hirayama, Japan), a UV–Vis spectrophotometer (Thermo Scientific, US), micropipette (Human Diagnostic), a spirit lamp, volumetric flasks, erlenmeyer flasks, beakers, petri dishes, sterile cotton swabs, Wickerham cards, inoculating loops, forceps, a spinball, and a ruler. The materials used were orange peel waste eco-enzyme, pure cultures of *Staphylococcus aureus* and *Escherichia coli*, chloramphenicol antibiotic disks (30 µg), Nutrient Agar medium, 1% H_2SO_4, 1% $BaCl_2$, 0.85% NaCl, and sterile distilled water.

Research Procedures

1. Preparation of Orange Peel Eco-Enzyme

All materials were measured using a ratio of 1:3:10, which corresponded to 120 g of palm sugar, 360 g of orange peel waste, and 1200 mL of clean water. The orange peels were cut into small pieces, and the palm sugar was finely ground. Water and palm sugar were placed into a glass jar and mixed until the sugar dissolved. The chopped orange peels were then added. The jar was tightly sealed and left to undergo fermentation for three months. To release fermentation gases, the container was opened daily during the first two weeks, every three days during the third week, and once a week during the following weeks. After three months, the organic residues were filtered out, and the remaining liquid was stored in a clean container protected from direct sunlight. Organoleptic evaluation included color, odor, pH, and volume of the product obtained.

2. Preparation of Equipment, Media, Bacterial Cultures, and 0.5 McFarland Standard

All equipment, culture media, and NaCl solution were sterilized using an autoclave. Bacterial cultures were refreshed on Nutrient Agar and used at 24 hours of incubation. A 0.5 McFarland standard was prepared by mixing H_2SO_4 and $BaCl_2$ solutions in appropriate proportions and then verified using a UV–Vis spectrophotometer at a wavelength of 625 nm. An absorbance value of 0.081 was obtained. The bacterial suspensions were prepared in NaCl solution and adjusted to match the turbidity of the 0.5 McFarland standard. The turbidity of a 0.5 McFarland standard corresponds to approximately 1.5×10^8 CFU/mL.

3. Antibacterial Activity Assay

The orange peel eco-enzyme was diluted with sterile distilled water to obtain concentrations of 70%, 80%, 90%, and 100%. Bacterial suspensions equivalent to the 0.5 McFarland standard were evenly spread over the entire surface of Nutrient Agar plates. Blank disks were loaded with 10 µL of eco-enzyme solution at concentrations of 70%, 80%, 90%, and 100% using a micropipette. Each impregnated disk was then placed on the surface of the Nutrient Agar, together with a chloramphenicol disk (30 µg) as a positive control and a blank disk loaded with sterile distilled water as a negative control. Six replicates were performed, as determined using Federer’s formula. All Petri dishes were incubated for 18 and 24 hours at 35 °C.

4. Statistical Data Analysis

The diameters of inhibition zones were analyzed using SPSS software. Data normality was assessed using the Shapiro–Wilk test. Differences among concentrations within the same bacterial species were analyzed using one-way ANOVA for normally distributed data, while non-normally distributed data were analyzed using the Kruskal–Wallis test. Differences between the two bacterial species were analyzed using an independent *t*-test or the Mann–Whitney test with IBM SPSS version 25 at a 95% confidence level. Antibacterial activity categories were determined based on the classification proposed by Irianto et al. (2023), as presented in the table below.

Table 1. Classification of Antibacterial Activity Strength

Diameter of Inhibition Zone (mm)	Category
≥20	Very strong
10 – 19	Strong
5 – 9	Moderate
<5	Weak

(Irianto *et al.*, 2023)

A summary of the experimental procedures is illustrated in the following diagram.

Figure 1. Experimental procedures

3. RESULTS AND DISCUSSION

The obtained eco-enzyme was evaluated organoleptically, and the characteristics of the eco-enzyme solution were recorded as follows.

Table 2. Characteristics of the Orange Peel Eco-Enzyme Solution

Color	Aroma	pH	Volume
Light brown	Strong characteristic citrus odor	4	700 mL

The results of the study, in the form of inhibition zone diameters, are presented in Table 3 below.

Table 3. Mean Inhibition Zone Diameters of Orange Peel Eco-Enzyme against Bacteria

Concentration (%)	**Mean Inhibition Zone Diameter (mm) at Incubation Time**					
	18-hours			24-hours		
	Staphylococcus aureus	*E.coli*	Category	*Staphylococcus aureus*	*E.coli*	Category
70	6.35	6.63	moderate	6.35	6.55	Moderate
80	6.58	6.97	moderate	6.45	6.77	Moderate
90	7.52	7.25	moderate	6.80	6.98	Moderate
100	7.73	8.36	moderate	7.23	7.57	Moderate
Chloramphenicol	34.17	28.78	sensitive	33.86	28.50	sensitive
Sterile aquadest	0	0		0	0	

When presented in graphical form, the results of the study are shown in the following figure.

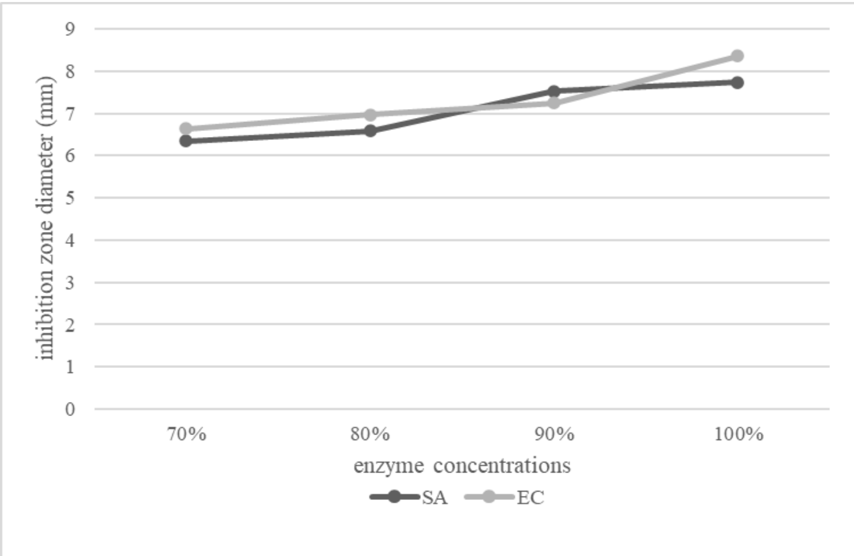


Figure 2. Comparison of the Mean Inhibition Zone Diameters of Orange Peel Eco-Enzyme at 18-Hour Incubation (SA = *Staphylococcus aureus*, EC = *Escherichia coli*)

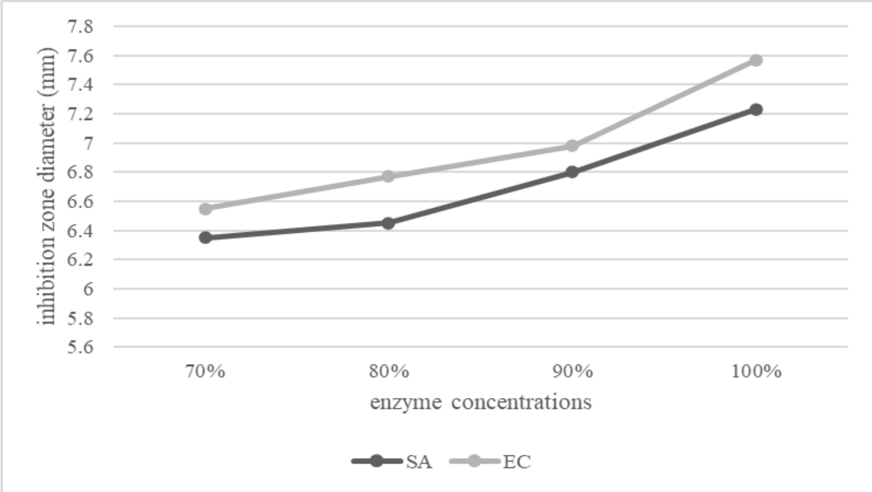
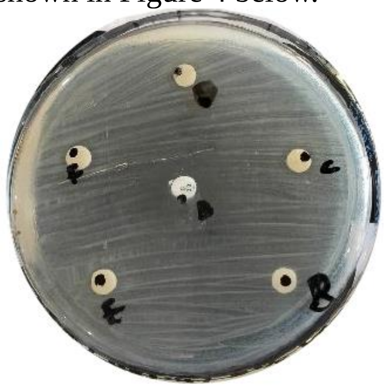


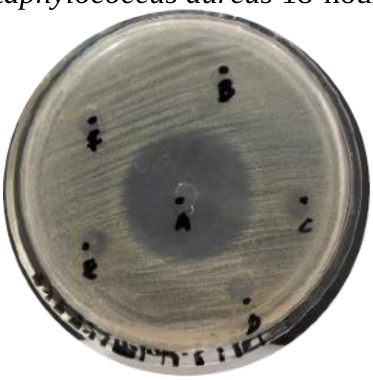
Figure 3. Comparison of the Mean Inhibition Zone Diameters of Orange Peel Eco-Enzyme at 24-Hour Incubation (SA = *Staphylococcus aureus*, EC = *Escherichia coli*)

Images of the observed inhibition zone diameters are shown in Figure 4 below.

Staphylococcus aureus 18-hours



Escherichia coli 18-hours



Staphylococcus aureus 24-hours

Escherichia coli 24-hours

Figure 4. Inhibition Zone Diameters of Orange Peel Eco-Enzyme (A = chloramphenicol; B = distilled water; C = 70%; D = 80%; E = 90%; F = 100%)

To determine differences in antibacterial activity among eco-enzyme concentrations, the Kruskal–Wallis test was applied, as the normality test indicated that the data were not normally distributed. The results of the test are presented in the following table.

Table 4. The Kruskal–Wallis Test for Eco-Enzyme Concentration Variables in Two Bacterial Species

Bacteria	**Significance value at incubation time**	
	18-hours	24-hours
Staphylococcus aureus	0.007*	0.070
Escherichia coli	0.024*	0.037*

*indicates a statistically significant difference among the tested groups ($p < 0.05$)

As the test results indicated differences among the tested groups (except for *Staphylococcus aureus* at 24 hours), the Mann–Whitney test was performed to identify which groups showed statistically significant differences. The results of the statistical analysis are presented in the following table.

Table 5. The Mann–Whitney Test among Eco-Enzyme Concentration Groups

Bacteria	**Compared groups**	**Significance value for incubation temperature**	
		18-hours	24-hours
Staphylococcus aureus	100% vs 90%	0.685	
	100% vs 80%	0.050*	
	100% vs 70%	0.004*	
	90% vs 80%	0.039*	
	90% vs 70%	0.013*	
	80% vs 70%	0.180	
Escherichia coli	100% vs 90%	0.146	0.124
	100% vs 80%	0.035*	0.032*
	100% vs 70%	0.010*	0.014*
	90% vs 80%	0.459	0.931
	90% vs 70%	0.099	0.113
	80% vs 70%	0.139	0.176

*indicates a statistically significant difference among the tested groups ($p < 0.05$)

Comparisons among concentrations at 18 hours indicated no significant difference between the 100% and 90% concentrations. This finding suggests that 90% orange peel eco-enzyme is the optimal concentration for inhibiting *Staphylococcus aureus*.

For *Escherichia coli*, significant differences in the antibacterial activity of orange peel eco-enzyme among concentrations were observed at both incubation times. Comparisons among concentrations showed that at both 18 and 24 hours there were significant differences between the 100% and 80% concentrations and between the 100% and 70% concentrations, whereas no significant differences were found among the other concentrations. These results indicate that 100% orange peel eco-enzyme is the most effective concentration for inhibiting *Escherichia coli*.

Overall, at a 95% confidence level (α = 0.05), it can be concluded that the eco-enzyme exhibits significant antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*, with effectiveness increasing as the concentration increases. These findings are consistent with the study by Irianto et al., which reported that higher eco-enzyme concentrations result in stronger antibacterial activity (Irianto *et al.*, 2023).

The antibacterial activity of orange peel eco-enzyme against *Staphylococcus aureus* and *Escherichia coli* is strongly associated with the presence of secondary metabolites and organic acids produced during the fermentation of organic materials, particularly the orange peel used in this study. The fermentation process generates compounds such as acetic acid, lactic acid, ethanol, and various hydrolytic enzymes, which collectively contribute to the antibacterial properties of the eco-enzyme (Arifah and Prima, 2025). Organic acid compounds act by lowering the environmental pH, thereby disrupting the stability of bacterial cell membranes and causing denaturation of intracellular proteins. This mechanism is particularly effective against Gram-negative bacteria such as *Escherichia coli*, which possess a lipopolysaccharide layer that is sensitive to pH changes. In contrast, phenolic compounds and flavonoids derived from the orange peel fermentation substrate are capable of damaging the cell wall, inhibiting protein synthesis, and interfering with bacterial enzyme activity, thereby enhancing the antibacterial effect against Gram-positive bacteria such as *Staphylococcus aureus*. The increased antibacterial effectiveness at higher concentrations (≥80%) indicates that the levels of active compounds in the eco-enzyme rise with increasing concentration, resulting in stronger inhibitory effects on bacterial growth.

These findings are consistent with those of Arifah and Prima (2025), who reported that eco-enzymes exhibit significant antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*, primarily due to the synergistic effects of organic acids and secondary metabolites produced during fermentation. Other studies have also shown that orange peel–based eco-enzymes contain high levels of acetic acid and phenolic compounds, which contribute to antibacterial effects by disrupting membrane permeability and causing protein denaturation. Thus, the antibacterial effectiveness of the eco-enzyme observed in this study can be explained by the bioactive compounds generated during fermentation and their ability to create acidic conditions and damage bacterial cell integrity. Higher concentrations and longer incubation times increase the likelihood of interactions between active compounds and bacterial structural components, resulting in larger inhibition zones.

The obtained data were statistically analyzed to determine whether there were differences in the antibacterial activity of the eco-enzyme between the two bacterial species. The normality test indicated that the data were not normally distributed; therefore, the Mann–Whitney test was used to assess differences in inhibition zone diameters between the bacteria at both 18 and 24 hours of incubation, as shown in Table 4 below.

Table 6. The Mann–Whitney Test between *Staphylococcus aureus* and *Escherichia coli* at Two Incubation Times

Incubation times	Significance value	Interpretation
18-hours	0.217	There was no significant difference in antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* at 18-hours of incubation
24-hours	0.062	There was no significant difference in antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* at 24-hours of incubation

Table 6 shows that there is no significant difference in the antibacterial activity of orange peel eco-enzyme against *Staphylococcus aureus* compared with *Escherichia coli*, at both 18 and 24 hours of incubation. These findings indicate that the eco-enzyme exhibits a relatively balanced antibacterial potential against both Gram-positive and Gram-negative bacteria, despite their differences in cell wall structure. This effect is likely attributable to the chemical properties of the eco-enzyme, which contains organic acids and phenolic compounds capable of penetrating the cell wall layers of both bacterial types. Organic acids such as acetic acid and lactic acid lower the environmental pH, leading to hydrogen ion dissociation that disrupts membrane stability and bacterial cellular metabolism. Meanwhile, phenolic compounds can bind to membrane proteins, causing cytoplasmic leakage in both *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) (Liang *et al.*, 2022; Lobiuc *et al.*, 2023). Therefore, although these two bacterial species are structurally different, their responses to eco-enzyme exposure are relatively similar ($p > 0.05$).

Subsequently, the Mann–Whitney test was conducted to determine whether there were differences in antibacterial activity between incubation times for each bacterial species, as shown in Table 7 below.

Table 7. The Mann–Whitney Test between 18- and 24-Hour Incubation Times in Two Bacterial Species

Bacteria	Significance value	Intepretation
Staphylococcus aureus	0.197	There was no significant difference in antibacterial activity against *Staphylococcus aureus* between 18 and 24 hours of incubation.
Escherichia coli	0.320	There was no significant difference in antibacterial activity against *Escherichia coli* between 18 and 24 hours of incubation.

Table 7 shows that the antibacterial activity of the orange peel eco-enzyme does not differ significantly between incubation times, either against *Staphylococcus aureus* or *Escherichia coli*. This indicates that increasing the incubation time from 18 to 24 hours did not significantly affect the resulting inhibition zone diameters. The antibacterial effect of the eco-enzyme likely reached an optimal level within the first 18 hours; therefore, extending the incubation time did not produce a significant increase in inhibitory activity.

According to Irianto *et al.* (2023), sweet orange peel eco-enzyme is capable of inhibiting the growth of *Staphylococcus aureus* due to the presence of secondary metabolites such as alkaloids, polyphenols, flavonoids, and saponins. These secondary metabolites inhibit bacterial growth through multiple mechanisms. Alkaloids exert antibacterial activity by disrupting the peptidoglycan components of bacterial cells, preventing proper cell wall formation and leading to cell death. Polyphenols are able to reduce intracellular ATP levels, induce bacterial cell membrane depolarization, decrease bacterial protein content, and cause cytoplasmic leakage. Flavonoids, due to their lipophilic properties, damage the structure of the cell membrane, thereby inhibiting bacterial growth. Saponins have the ability to degrade the cell wall, followed by disruption of the cytoplasmic membrane and membrane proteins, resulting in leakage of bacterial cell contents and ultimately cell death.

4. CONCLUTION

The results of this study indicate that orange peel eco-enzyme exhibits antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*, and that there are differences in antibacterial effectiveness among eco-enzyme concentrations. Different incubation times (18 and 24 hours) did not result in significant differences in the antibacterial effectiveness of orange peel eco-enzyme against either *Staphylococcus aureus* or *Escherichia coli*. The orange peel eco-enzyme shows comparable potential to inhibit the growth of both bacterial species, suggesting that it can be further developed as an environmentally friendly disinfectant.

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