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Impact Sugar Type, Fermentation Duration, and Concentration of Eco-Enzyme on pH, TDS, and Antibacterial Activity

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Abstract: Eco-enzymes produced from fruit and vegetable waste are increasingly used for waste valorization and natural disinfection, but the combined effects of sugar type, fermentation duration and dilution on their quality remain unclear. This study examined eco-enzymes from mixed fruit peels prepared with granulated sugar, brown sugar and molasses, fermented for 1, 2 and 3 months, and diluted to 20%, 10%, 1%, 0.2% and 0.1%. Physicochemical properties (pH, TDS) and antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* were measured; data were analysed by one-way ANOVA with Tukey's test and Pearson correlation. Eco-enzymes were strongly acidic (≈3.5–3.9) at 20–10% and approached neutrality at 0.1%, with molasses-based formulations maintaining the lowest pH at high dilutions. TDS showed fermentation-time-dependent trends that differed between concentrated and dilute solutions. No inhibition was observed in month 1; activity appeared after ≥2 months and was highest at 3 months, with three-month molasses eco-enzymes at 20% giving the largest inhibition zones and retaining activity at 0.1–0.2%. These findings identify molasses and extended fermentation as key levers to improve eco-enzyme performance as low-cost natural antibacterial agents.

Keywords: Antibacterial activity; Eco-enzyme; Fermentation duration; Sugar type.

Introduction

Eco-enzymes have emerged as a promising approach for organic waste management while supporting the principles of a circular economy. Organic waste, particularly fruit and vegetable residues, constitutes the largest proportion of municipal waste and can generate methane emissions that contribute to global warming if not properly managed (Dhar et al., 2025; Agustin & Widyaningrum, 2026). Eco-enzymes convert organic waste into value-added products through a simple fermentation process using organic waste, sugar, and water at a ratio of 3:1:10 (Barman et al., 2022; Setiawan et al., 2025). The resulting product has been widely utilized as an organic fertilizer, antibacterial agent, wastewater treatment additive, and natural disinfectant, demonstrating its potential to reduce organic waste while promoting environmental sustainability (Gumilar et al., 2025; Vidalia et al., 2023; Al-Astani Tengku Din et al., 2025; Barman et al., 2022; Dhar et al., 2025).

The quality of eco-enzymes is influenced by several factors, particularly sugar type, organic substrate, and fermentation duration. During fermentation, indigenous microorganisms, mainly lactic acid bacteria and acetic acid bacteria, convert carbohydrates into organic acids and other bioactive compounds through sequential biochemical processes (Suriani et al., 2023; Prakoso et al., 2025).

Fermentation generally produces alcohol during the first month, organic acids during the second month, and enzyme-rich products after approximately three months (Barman et al., 2022). Sugar type also plays an important role in determining fermentation efficiency, with molasses often producing better-quality eco-enzymes than refined or brown sugar because of its higher nutrient content and indigenous microorganisms (Putri et al., 2024). Moreover, prolonged fermentation has been reported to enhance antibacterial activity through the accumulation of organic acids and secondary metabolites, including phenolic compounds, flavonoids, alkaloids, terpenoids, and tannins (Mavani et al., 2020; Tallei et al., 2023; Setiawan et al., 2025).

The physicochemical properties of eco-enzymes, particularly pH and total dissolved solids (TDS), are widely used as indicators of fermentation quality. Successful fermentation generally produces eco-enzymes with a pH below 4 because of the accumulation of organic acids, especially acetic acid (Abdullah et al., 2023; Permatananda & Pandit, 2023; Vidalia et al., 2023). Meanwhile, TDS reflects the concentration of dissolved organic compounds and minerals formed during fermentation and varies according to the substrate and fermentation conditions (Maharani & Lusiani, 2023; Karimah & Lusiani, 2024). In addition to their physicochemical characteristics, eco-enzymes exhibit antibacterial activity against both Gram-positive and Gram-negative bacteria through the combined action of organic acids, hydrolytic enzymes, and bioactive compounds (Vidalia et al., 2023; Mavani et al., 2020; Tallei et al., 2023). Previous studies have also demonstrated comparable antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*, although the effectiveness varies depending on the raw materials and fermentation conditions (Cindana et al., 2025; Rinihapsari et al., 2026; Agustin & Widyaningrum, 2026).

Although eco-enzyme research has increased substantially, studies evaluating the combined effects of sugar type, fermentation duration, and dilution concentration on eco-enzyme quality remain limited. Previous studies have generally examined these factors separately, while information regarding their combined influence on physicochemical characteristics (pH and TDS) and antibacterial activity is still insufficient (Mavani et al., 2020; Primarista et al., 2026; Agustin & Widyaningrum, 2026). Therefore, this study aimed to evaluate the effects of different sugar types, fermentation durations, and dilution concentrations on the pH, TDS, and antibacterial activity of eco-enzymes against *Staphylococcus aureus* and *Escherichia coli*. The findings are expected to provide scientific information for optimizing eco-enzyme production from organic waste as an environmentally friendly antibacterial agent.

Materials and Methods

Tools and Materials

The main instruments used in this study included an incubator (Memmert IN55), hot plate magnetic stirrer (JOANLAB SH3), analytical balance (SHIMADZU TW223L), digital pH meter, digital TDS meter, and standard laboratory glassware. The materials consisted of granulated sugar, brown sugar, molasses, mixed fruit peels (dragon fruit, orange, melon, watermelon, guava, and apple), *Staphylococcus aureus* and *Escherichia coli* cultures, pH buffer solutions (4.00, 7.00, and 10.00), sodium hypochlorite (Onemed), nutrient agar (Oxoid), nutrient broth (HiMedia), Whatman No. 1 filter paper, and distilled water.

This study evaluated nine eco-enzyme treatments consisting of three sugar types (granulated sugar, brown sugar, and molasses) combined with three fermentation periods (1, 2, and 3 months). The resulting eco-enzyme filtrates were diluted with distilled water to concentrations of 20%, 10%, 1%, 0.2%, and 0.1% prior to antibacterial testing. The type of organic waste was maintained constant throughout the experiment.

Eco-Enzyme Production

Eco-enzymes were produced by fermenting 300 g of mixed fruit peels with 100 g of sugar and 1,000 mL of water in plastic containers following a ratio of 3:1:10. Three sugar sources, namely granulated sugar, brown sugar, and molasses, were used as fermentation substrates. The fermentation process was conducted for 1, 2, and 3 months at room temperature. After each fermentation period, the liquid was filtered using

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Whatman No. 1 filter paper and stored in sterile glass bottles for subsequent analyses (Hanifah et al., 2022).

Determination of pH and TDS

The pH and total dissolved solids (TDS) values of each eco-enzyme sample were measured using a calibrated digital pH meter and digital TDS meter, respectively. The probe was immersed in the sample until a stable reading was obtained, and each measurement was performed in triplicate. The pH values and TDS concentrations (ppm) were recorded as the mean of three measurements (Singkam, 2020).

Antibacterial Activity Assay

Antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* was determined using the disc diffusion method. All media and laboratory equipment were sterilized by autoclaving at 121°C and 15 psi for 15 min before use. Bacterial cultures were rejuvenated on nutrient agar and subsequently grown in nutrient broth at 37°C for 24 h. Sterile paper discs were immersed for 20 min in distilled water (negative control), 2.5% sodium hypochlorite (positive control), or the respective eco-enzyme solutions. The discs were then placed on nutrient agar plates previously inoculated with the test bacteria and incubated at 37°C for 24 h. Antibacterial activity was expressed as the inhibition zone diameter (mm), calculated from the diameter of the clear zone after subtracting the diameter of the paper disc (Primarista et al., 2023).

Data Analysis

All experiments were performed in triplicate, and the results are presented as mean ± standard deviation (SD). Data normality and homogeneity were evaluated using the Shapiro–Wilk and homogeneity tests, respectively. The effects of sugar type and fermentation duration on pH, TDS, and inhibition zone diameter were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test at a significance level of 5%. Pearson correlation analysis was performed to evaluate the relationships among the observed variables (Primarista et al., 2026).

Results and Discussion

Effect of Sugar Type and Fermentation Time on pH

The pH values of the eco-enzymes (Table 1) remained acidic (3.47–3.99) at concentrations of 20–10% and gradually increased as the dilution increased, reaching 4.86–6.49 at the 0.1% concentration. These results indicate that the acidity of eco-enzymes decreased proportionally with dilution. Similar findings have been reported in previous studies, which showed that well-fermented eco-enzymes generally exhibit pH values below 4 due to the accumulation of organic acids produced during fermentation (Barman et al., 2022; Dhar et al., 2025; Agustin & Widyaningrum, 2026). Comparable pH values were also reported for eco-enzymes produced from Kintamani orange peels (3.74) (Permatananda & Pandit, 2023) and tempeh waste (3.05–3.25) (Cundari et al., 2024). The low pH is mainly attributed to the production of acetic acid and lactic acid by microbial fermentation, whereas dilution reduces the concentration of these organic acids, resulting in a gradual increase in pH (Vidalia et al., 2023; Al-Astani Tengku Din et al., 2025; Gumilar et al., 2025; Prakoso et al., 2025).

Differences among sugar types became more evident at higher dilution levels. Eco-enzymes produced with molasses maintained lower pH values than those prepared with granulated and brown sugar, particularly at the 0.1% concentration. This finding agrees with Cundari et al. (2024), who reported that molasses produced more acidic eco-enzymes than other sugar sources. The higher acidity of molasses-based eco-enzymes is likely associated with its higher mineral content, indigenous microorganisms, and naturally occurring organic acids that promote fermentation and organic acid production (Suriani et al., 2023; Vidalia et al., 2023; Prakoso et al., 2025).

Similarly, Abdullah et al. (2023) demonstrated that different sugar sources significantly affected acetic acid production during fermentation. In contrast, Maharani and Lusiani (2023) reported that increasing the concentration of tempeh yeast increased the pH of eco-enzymes, suggesting that fermentation characteristics are influenced not only by sugar type but also by substrate composition and microbial communities. The relatively higher pH observed in eco-enzymes produced with granulated sugar may be related to its lower mineral and organic acid contents compared with molasses and brown sugar (Suriani et al., 2023; Vidalia et al., 2023).

Table 1. pH Values of Eco-Enzymes Produced Using Different Sugar Types, Fermentation Durations, And Dilution Concentrations

Sugar Type	Fermentation Duration	pH				
		20%	10%	1%	0.20%	0.10%
Granulated sugar	1	3.62 ± 0.03^{A,Bb}	3.83 ± 0.02^{Bb}	4.53 ± 0.01^{Bc}	5.78 ± 0.01^{Bc}	6.49 ± 0.01^{Cc}
Granulated sugar	2	3.57 ± 0.02^{Aa}	3.64 ± 0.03^{Aa}	4.04 ± 0.04^{Aa}	4.62 ± 0.01^{Aa,b}	5.28 ± 0.02^{Bb}
Granulated sugar	3	3.67 ± 0.01^{Ba}	3.71 ± 0.02^{Aa}	4.11 ± 0.02^{Ab}	4.58 ± 0.02^{Ab}	5.14 ± 0.02^{Ac}
Brown sugar	1	3.47 ± 0.02^{Aa}	3.56 ± 0.03^{Aa}	3.92 ± 0.01^{Aa}	4.54 ± 0.02^{Aa}	5.32 ± 0.02^{Ca}
Brown sugar	2	3.63 ± 0.01^{Ba}	3.64 ± 0.02^{Ba}	4.14 ± 0.02^{Ca}	4.61 ± 0.02^{Ba}	5.21 ± 0.02^{Ba}
Brown sugar	3	3.62 ± 0.02^{Ba}	3.67 ± 0.02^{Ba}	3.98 ± 0.02^{Ba}	4.48 ± 0.02^{Aa}	4.97 ± 0.01^{Ab}
Molasses	1	3.86 ± 0.01^{Ac}	3.87 ± 0.02^{Ab}	4.06 ± 0.04^{Ab}	4.61 ± 0.02^{Ab}	5.44 ± 0.02^{Cb}
Molasses	2	3.98 ± 0.02^{Bb}	4.00 ± 0.02^{Bb}	4.28 ± 0.05^{Bb}	4.67 ± 0.02^{Bb}	5.22 ± 0.03^{Ba}
Molasses	3	3.99 ± 0.02^{Bb}	3.98 ± 0.02^{Bb}	4.17 ± 0.03^{A,Bb}	4.57 ± 0.02^{Ab}	4.86 ± 0.04^{Aa}

*Data are the average of 3 replications and are followed by different letters indicating significant differences in the Tukey's Honest Significant Differences (HSD) test with (p<0.05). Capital letters indicate significant differences between eco-enzymes and sugar variations, while lowercase letters indicate significant differences between eco-enzymes and fermentation duration variations.

Table 2. TDS Values of Eco-Enzymes Produced Using Different Sugar Types, Fermentation Durations, And Dilution Concentrations

Sugar Type	Fermentation Duration	TDS				
		20%	10%	1%	0.20%	0.10%
Granulated sugar	1	326.00 ± 3.74^{Aa}	256.67 ± 1.70^{Aa}	91.67 ± 1.70^{Ca}	25.33 ± 2.05^{Aa}	16.33 ± 1.70^{Aa}
Granulated sugar	2	455.67 ± 2.45^{Ba}	266.67 ± 2.05^{Ba}	77.33 ± 1.25^{Ba}	27.00 ± 0.94^{Aa}	16.67 ± 1.25^{Aa}
Granulated sugar	3	505.33 ± 4.97^{Ca}	306.33 ± 2.05^{Ca}	68.67 ± 1.70^{Aa}	26.00 ± 0.47^{Aa}	17.00 ± 2.16^{Aa}
Brown sugar	1	564.00 ± 2.05^{Ab}	366.67 ± 2.87^{Ab}	116.33 ± 1.70^{Cb}	31.33 ± 2.16^{Ab}	16.67 ± 1.25^{Aa}
Brown sugar	2	592.67 ± 1.89^{Bb}	379.67 ± 1.25^{Bb}	105.67 ± 3.30^{Bc}	28.00 ± 2.16^{Aa}	16.67 ± 1.25^{Aa}
Brown sugar	3	567.67 ± 1.25^{Ab}	367.67 ± 1.70^{Ab}	71.67 ± 1.25^{Aa}	32.67 ± 1.25^{Ab}	18.67 ± 1.25^{Aa}
Molasses	1	1093.00 ± 3.86^{Bc}	768.33 ± 1.70^{Cc}	154.67 ± 1.25^{Cc}	40.33 ± 0.82^{A,Bc}	27.00 ± 2.16^{A,Bb}
Molasses	2	649.33 ± 3.68^{Ac}	436.67 ± 2.05^{Ac}	96.67 ± 1.70^{Ab}	37.33 ± 1.25^{Ab}	22.33 ± 1.70^{Ab}
Molasses	3	1107.00 ± 2.45^{Cc}	665.33 ± 1.25^{Bc}	137.33 ± 2.05^{Bb}	43.00 ± 2.45^{Bc}	31.67 ± 1.70^{Bb}

*Data are the average of 3 replications and are followed by different letters indicating significant differences in the Tukey's Honest Significant Differences (HSD) test with (p<0.05). Capital letters indicate significant differences between eco-enzymes and sugar variations, while lowercase letters indicate significant differences between eco-enzymes and fermentation duration variations.

The Effect of Sugar Type and Fermentation Time on TDS

The TDS values of eco-enzymes were significantly affected by sugar type, fermentation duration, and dilution concentration (Table 2). Molasses-based eco-enzymes consistently exhibited the highest TDS values, followed by brown sugar and granulated sugar, across all fermentation periods and dilution levels. At 20% concentration, TDS ranged from approximately 650–1100 mg/L for molasses, 560–600 mg/L for brown sugar, and 300–500 mg/L for granulated sugar. This pattern reflects the inherent composition of molasses, which contains abundant minerals, organic acids, and non-sucrose compounds that contribute to dissolved solids during fermentation (Dhar et al., 2025; Agustin & Widyaningrum, 2026). Previous studies similarly reported that molasses produces eco-enzymes with higher dissolved solids because it supplies additional nutrients and minerals that support microbial metabolism throughout fermentation (Barman et al., 2022; Gumilar et al., 2025; Vidalia et al., 2023).

Fermentation duration also influenced TDS values. In eco-enzymes prepared with granulated and brown sugar, TDS generally increased from the first to the third month at higher concentrations (20–10%), suggesting continued degradation of organic substrates into soluble metabolites. This observation agrees with reports that prolonged fermentation enhances enzymatic hydrolysis of carbohydrates, proteins, and lipids, thereby increasing dissolved organic compounds (Maharani & Lusiani, 2023; Kamaliya & Lusiani, 2023; Suriani et al., 2023). In contrast, diluted solutions (1–0.1%) tended to exhibit lower TDS after longer fermentation, indicating that mineralization, precipitation of insoluble compounds, and conversion of dissolved carbon into CO_2 became more dominant during the later fermentation stages (Putri et al., 2024; Permatananda & Pandit, 2023; Al-Astani Tengku Din et al., 2025).

As expected, TDS decreased progressively with increasing dilution because dissolved solids were proportionally reduced in the diluted solution. Similar trends have been reported in eco-enzyme studies used for wastewater treatment, where dilution substantially decreased dissolved solids despite the relatively high organic content of the original eco-enzyme (Vidalia et al., 2023; Putri et al., 2024). Overall, the present results demonstrate that sugar type primarily determines the initial dissolved solid content, whereas fermentation duration modifies TDS through microbial degradation and metabolite production.

Antibacterial Activity against *Staphylococcus aureus*

The antibacterial activity of eco-enzymes against *Staphylococcus aureus* was strongly influenced by fermentation duration, sugar type, and dilution concentration (Table 3). No inhibition zone was observed after one month of fermentation, whereas antibacterial activity appeared after two months and reached its maximum after three months. This finding agrees with previous studies demonstrating that prolonged fermentation promotes the accumulation of antimicrobial compounds, particularly organic acids and hydrolytic enzymes (Barman et al., 2022; Mavani et al., 2020; Islam et al., 2022; Tallei et al., 2023).

Among the sugar treatments, molasses consistently produced the greatest inhibition zones, particularly after three months of fermentation, reaching approximately 5–6 mm at 20% concentration. Brown sugar showed intermediate activity, whereas granulated sugar produced the lowest inhibition zones. These results are consistent with previous reports indicating that molasses provides superior nutrients for fermentative microorganisms, resulting in greater production of antimicrobial metabolites, including acetic acid and other bioactive compounds (Primarista et al., 2026; Agustin & Widyaningrum, 2026; Gumilar et al., 2025; Prakoso et al., 2025). The richer mineral and nutrient composition of molasses compared with purified sucrose further supports microbial growth and metabolite synthesis during fermentation (Suriani et al., 2023; Setiawan et al., 2025).

Table 3. Inhibition Zone of Eco-Enzymes Against *Staphylococcus Aureus*

Sugar Type	Fermentation Duration	Inhibition zone against *Staphylococcus aureus* (mm)				
		20%	10%	1%	0.20%	0.10%
Granulated sugar	1	$0.00 \pm 0.00^{A*}$	$0.00 \pm 0.00^{A*}$	$0.00 \pm 0.00^{A*}$	$0.00 \pm 0.00^{A*}$	$0.00 \pm 0.00^{A*}$
Granulated sugar	2	4.26 ± 0.03^{Ba}	3.47 ± 0.02^{Bb}	2.84 ± 0.04^{Bb}	1.39 ± 0.06^{Ba}	0.63 ± 0.12^{Ba}
Granulated sugar	3	4.25 ± 0.05^{Ba}	3.91 ± 0.04^{Ca}	2.58 ± 0.29^{Ba}	2.11 ± 0.05^{Ca}	1.13 ± 0.07^{Ca}
Brown sugar	1	$0.00 \pm 0.00^{A*}$	$0.00 \pm 0.00^{A*}$	$0.00 \pm 0.00^{A*}$	$0.00 \pm 0.00^{A*}$	$0.00 \pm 0.00^{A*}$
Brown sugar	2	4.23 ± 0.06^{Ba}	3.47 ± 0.06^{Bb}	2.83 ± 0.08^{Bb}	1.37 ± 0.05^{Ba}	0.68 ± 0.09^{Ba}
Brown sugar	3	4.79 ± 0.05^{Cb}	3.85 ± 0.04^{Ca}	3.55 ± 0.03^{Cb}	3.42 ± 0.03^{Cb}	3.07 ± 0.08^{Cb}
Molasses	1	$0.00 \pm 0.00^{A*}$	$0.00 \pm 0.00^{A*}$	$0.00 \pm 0.00^{A*}$	$0.00 \pm 0.00^{A*}$	$0.00 \pm 0.00^{A*}$
Molasses	2	4.37 ± 0.05^{Ba}	3.16 ± 0.05^{Ba}	2.31 ± 0.02^{Ba}	2.02 ± 0.02^{Bb}	1.39 ± 0.02^{Bb}
Molasses	3	5.93 ± 0.05^{Cc}	5.85 ± 0.03^{Cb}	5.59 ± 0.07^{Cc}	5.30 ± 0.03^{Cc}	4.75 ± 0.05^{Cc}

*Data are the average of 3 replications and are followed by different letters indicating significant differences in the Tukey's Honest Significant Differences (HSD) test with ($p<0.05$). Capital letters indicate significant differences between eco-enzymes and sugar variations, while lowercase letters indicate significant differences between eco-enzymes and fermentation duration variations. An asterisk indicates no significant difference.

A clear concentration-dependent response was also observed. Higher eco-enzyme concentrations produced larger inhibition zones, whereas antibacterial activity gradually

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decreased as dilution increased. Similar dose-dependent antibacterial effects have been reported in previous studies, where higher eco-enzyme concentrations contained greater amounts of organic acids, flavonoids, phenolics, and hydrolytic enzymes responsible for bacterial inhibition (Mavani et al., 2020; Oktiansyah & Habısukan, 2026; Cindana et al., 2025). Although the inhibition zones obtained in this study (approximately 4–6 mm) were categorized as weak to moderate (Permatananda & Pandit, 2023), they demonstrate that eco-enzyme possesses measurable antibacterial activity even at relatively low concentrations. The smaller inhibition zones compared with some previous studies may be attributed to differences in eco-enzyme concentration, fermentation substrate, and antibacterial assay methods (Karimah & Lusiani, 2024; Agustin & Widyaningrum, 2026).

Table 4. Inhibition Zone of Eco-Enzymes Against *Escherichia coli*

Sugar Type	Fermentation Duration	Inhibition zone against *Escherichia coli* (mm)				
		20%	10%	1%	0.20%	0.10%
Granulated sugar	1	$0.00 \pm 0.00^{A*}$	$0.00 \pm 0.00^{A*}$	$0.00 \pm 0.00^{A*}$	$0.00 \pm 0.00^{A*}$	$0.00 \pm 0.00^{A*}$
Granulated sugar	2	4.86 ± 0.05^{Bb}	3.46 ± 0.05^{Bb}	3.10 ± 0.05^{Bb}	2.15 ± 0.03^{Ba}	1.39 ± 0.06^{Ba}
Granulated sugar	3	4.80 ± 0.05^{Bb}	3.74 ± 0.05^{Ca}	3.74 ± 0.04^{Ca}	3.24 ± 0.05^{Ca}	3.05 ± 0.04^{Ca}
Brown sugar	1	$0.00 \pm 0.00^{A*}$	$0.00 \pm 0.00^{A*}$	$0.00 \pm 0.00^{A*}$	$0.00 \pm 0.00^{A*}$	$0.00 \pm 0.00^{A*}$
Brown sugar	2	4.81 ± 0.03^{Cb}	3.47 ± 0.03^{Bb}	3.09 ± 0.07^{Bb}	2.15 ± 0.05^{Ba}	1.35 ± 0.06^{Ba}
Brown sugar	3	4.57 ± 0.04^{Ba}	4.40 ± 0.07^{Cb}	3.76 ± 0.03^{Ca}	3.50 ± 0.03^{Cb}	3.37 ± 0.07^{Cb}
Molasses	1	$0.00 \pm 0.00^{A*}$	$0.00 \pm 0.00^{A*}$	$0.00 \pm 0.00^{A*}$	$0.00 \pm 0.00^{A*}$	$0.00 \pm 0.00^{A*}$
Molasses	2	4.51 ± 0.07^{Ba}	3.26 ± 0.04^{Ba}	2.47 ± 0.05^{Ba}	2.05 ± 0.04^{Ba}	1.41 ± 0.06^{Ba}
Molasses	3	6.07 ± 0.06^{Cc}	5.75 ± 0.05^{Cc}	5.11 ± 0.05^{Cb}	4.62 ± 0.03^{Cc}	4.37 ± 0.05^{Cc}

*Data are the average of 3 replications and are followed by different letters indicating significant differences in the Tukey's Honest Significant Differences (HSD) test with ($p<0.05$). Capital letters indicate significant differences between eco-enzymes and sugar variations, while lowercase letters indicate significant differences between eco-enzymes and fermentation duration variations. An asterisk indicates no significant difference.

The antibacterial activity is likely associated with the combined effects of organic acids and bioactive compounds produced during fermentation. Acetic acid disrupts bacterial cell membrane integrity, while flavonoids such as hesperidin inhibit DNA gyrase, thereby interfering with bacterial DNA replication (Vidalia et al., 2023; Tallei et al., 2023). These mechanisms explain the enhanced antibacterial activity observed after prolonged fermentation and in molasses-based eco-enzymes.

Antibacterial Activity against *Escherichia coli*

Similar to the results obtained for *S. aureus*, eco-enzyme antibacterial activity against *Escherichia coli* depended on sugar type, fermentation duration, and dilution concentration (Table 4). No inhibition zone was detected after one month of fermentation, whereas antibacterial activity appeared after two months and increased markedly after three months. Molasses-based eco-enzyme fermented for three months exhibited the highest antibacterial activity, producing inhibition zones exceeding 6 mm at 20% concentration, followed by brown sugar and granulated sugar.

These findings agree with previous studies reporting that longer fermentation enhances the accumulation of antimicrobial metabolites responsible for inhibiting Gram-negative bacteria (Islam et al., 2022; Mavani et al., 2020; Primarista et al., 2026). Molasses has also been reported to improve fermentation efficiency because of its higher mineral and nutrient contents, thereby promoting greater production of organic acids and antimicrobial compounds (Agustin & Widyaningrum, 2026; Gumilar et al., 2025).

The inhibition zones decreased consistently with decreasing eco-enzyme concentration, indicating a typical concentration-dependent antibacterial response. Higher concentrations provide greater amounts of acetic acid, enzymes, and phenolic compounds capable of disrupting bacterial membranes and inhibiting cellular metabolism (Vidalia et al., 2023; Setiawan et al., 2025; Cindana et al., 2025). Interestingly, the antibacterial activity against *E. coli* was comparable to that observed against *S. aureus*, supporting previous reports that eco-enzymes exhibit broad-spectrum antibacterial properties against both Gram-positive and Gram-negative bacteria (Rinihapsari et al., 2026; Agustin & Widyaningrum, 2026). Differences in inhibition zones among studies are likely associated with variations in fermentation substrates, eco-enzyme concentration, fermentation duration, and antimicrobial testing methods.

Comparison of Activity against *Escherichia coli* and *Staphylococcus aureus*

The present study demonstrated that eco-enzymes exhibited comparable antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*, with inhibition zones against *E. coli* being slightly larger under most treatment combinations (Tables 3 and 4). Similar findings have been reported for orange peel eco-enzymes, which showed no significant difference in antibacterial activity between Gram-positive and Gram-negative bacteria, indicating the broad-spectrum antimicrobial potential of eco-enzymes (Rinihapsari et al., 2026). Likewise, Cindana et al. (2025) found that eco-enzymes derived from various fruit peels effectively inhibited both bacterial groups without significant differences in antibacterial activity.

The superior antibacterial performance of molasses-based eco-enzymes observed in this study is consistent with previous reports showing that molasses provides a more suitable substrate for microbial fermentation than granulated or brown sugar (Primarista et al., 2026; Agustin & Widyaningrum, 2026). Molasses contains abundant minerals, vitamins, amino acids, and indigenous microorganisms that enhance microbial metabolism and promote the production of organic acids and other antimicrobial metabolites during fermentation (Suriani et al., 2023; Gumilar et al., 2025; Prakoso et al., 2025). Consequently, eco-enzymes produced from molasses maintained antibacterial activity even at the lowest dilution (0.1%) after three months of fermentation.

The absence of antibacterial activity after one month of fermentation and the marked increase after two to three months indicate that antimicrobial metabolite production is closely associated with fermentation progress. During eco-enzyme fermentation, alcohol predominates in the early stage, whereas acetic acid and hydrolytic enzymes accumulate during the second and third months, resulting in greater antibacterial activity (Barman et al., 2022; Islam et al., 2022; Mavani et al., 2020). Furthermore, the concentration-dependent inhibition observed in this study agrees with previous reports that higher eco-enzyme concentrations contain greater amounts of organic acids, enzymes, flavonoids, and phenolic compounds capable of disrupting bacterial cell membranes and inhibiting cellular metabolism (Vidalia et al., 2023; Tallei et al., 2023; Oktiansyah & Habısukan, 2026). These findings suggest that molasses-based eco-enzymes fermented for three months represent the most effective formulation for broad-spectrum antibacterial applications.

Relationship between pH, TDS, and Antibacterial Activity

Correlation analysis demonstrated that pH was negatively associated with the inhibition zones of *S. aureus* (r = −0.357) and *E. coli* (r = −0.331), whereas TDS showed positive correlations with antibacterial activity (r = 0.292 and r = 0.256, respectively). These results indicate that lower pH and higher dissolved solids tend to enhance the antibacterial properties of eco-enzymes, although both relationships were classified as weak to moderate.

The negative relationship between pH and antibacterial activity is consistent with the accumulation of organic acids during fermentation. Acetic acid, citric acid, and other fermentation products lower the pH while simultaneously disrupting bacterial cell membranes and reducing intracellular pH, thereby inhibiting bacterial growth (Barman et al., 2022; Hanifah et al., 2022; Vidalia et al., 2023). However, the relatively weak correlation suggests that antibacterial activity is not determined solely by acidity. Previous studies have shown that hydrolytic enzymes, phenolic compounds, flavonoids, tannins, and other secondary metabolites also contribute substantially to the antimicrobial properties of eco-enzymes (Mawli et al., 2025; Setiawan et al., 2025).

Similarly, the positive correlation between TDS and antibacterial activity indicates that dissolved metabolites contribute to bacterial inhibition. Higher TDS reflects greater concentrations of organic acids, enzymes, minerals, and bioactive compounds generated during fermentation (Barman et al., 2022; Gumilar et al., 2025; Prakoso et al., 2025). Nevertheless, because TDS also includes nonbioactive minerals and inert dissolved substances, its relationship with antibacterial activity remained relatively weak. Comparable correlation patterns against both *S. aureus* and *E. coli* further support the broad-spectrum antibacterial characteristics of eco-enzymes (Suriani et al., 2023; Cindana et al., 2025).

Overall, these findings indicate that the antibacterial activity of eco-enzymes results from the combined effects of acidity, dissolved metabolites, hydrolytic enzymes, and secondary metabolites rather than from pH or TDS alone. Therefore, pH and TDS should be regarded as useful indicators of eco-enzyme quality but not as the sole predictors of antibacterial performance (Gumilar et al., 2025; Al-Astani Tengku Din et al., 2025; Setiawan et al., 2025).

Conclusion

This study demonstrates that sugar type, fermentation duration, and dilution concentration significantly influence the physicochemical characteristics and antibacterial activity of multi-fruit peel eco-enzymes. Molasses produced eco-enzymes with lower pH, higher TDS, and stronger antibacterial activity than brown sugar and granulated sugar, particularly after three months of fermentation. Antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* was only detected after at least two months of fermentation and increased with fermentation duration, while dilution progressively reduced antibacterial effectiveness. Correlation analysis further indicated that lower pH and higher TDS were associated with greater antibacterial activity, although these relationships were relatively weak, suggesting that antibacterial performance is determined by multiple bioactive compounds rather than physicochemical properties alone. These findings indicate that molasses fermented for three months provides the most effective formulation among the treatments evaluated and has potential as a natural antimicrobial product derived from organic waste. Future studies should characterize the metabolite composition of eco-enzymes, quantify major organic acids and bioactive compounds, evaluate broader microbial targets, and investigate the stability and practical application of eco-enzymes under real-use conditions.

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