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Sustainable Antibiofilm Strategy Using Fruit Waste: Eco-Enzyme from Pineapple Core and Lemon Peel Against *Streptococcus mutans* and *Candida albicans*

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ABSTRACT: Eco-enzyme is a versatile liquid produced by fermenting organic material in molasses solution and has some potential antimicrobial compounds. In this study, the organic materials utilized were pineapple cores and lemon peels, which are typically discarded. Oral microbes, particularly *Streptococcus mutans* and *Candida albicans*, often develop antimicrobial resistance through their defense mechanisms by forming a polymeric layer known as biofilm. This biofilm, composed of polysaccharides, protects microbes from exposure to antimicrobial agents, allowing them to adapt and survive. The objective of this study was to evaluate the antibiofilm properties of eco-enzymes derived from various organic materials. The assessment involved determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)/minimum fungicidal concentration (MFC) and conducting an antibiofilm assay. The eco-enzyme formulated with pineapple cores exhibited higher antimicrobial activity, with an MIC of 25% and an MBC of 50% against *Streptococcus mutans*. Additionally, the highest antibiofilm activity was observed in the eco-enzyme containing a specific organic matter combination, with values of 68526.23 ppm against *Streptococcus mutans* and 2235.30 ppm against *Candida albicans*. The IC50 value for all three eco-enzyme samples was analyzed using a one-way ANOVA statistical test, revealing significant inhibitory activity against biofilm formation by *Streptococcus mutans* (p=0.023) and *Candida albicans* (p=0.002).

Keywords: Biofilm; *Candida albicans*; Eco-Enzyme; *Streptococcus mutans*

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INTRODUCTION

The oral cavity is known for having the second highest diversity of microbiota, following the gastrointestinal tract, forming a complex ecosystem that includes various opportunistic pathogens capable of causing significant oral and dental diseases. Dental plaque, a biofilm that develops on both soft and hard oral tissues, is central to the pathogenesis of these conditions (Salehi et al., 2020). Statistics from Riset Kesehatan Dasar 2013 (Kementerian Kesehatan RI, 2013) and Survei Kesehatan Indonesia 2023 (Badan Kebijakan Pembangunan Kesehatan, 2023) reveal a dramatic rise in the prevalence of oral and dental problems in Indonesia, from 25.9% in 2013 to 56.9% in 2023. The most frequent complaints included toothaches, damaged teeth, and dental caries, which made up 86% of all cases.

Among the microbes found in the oral cavity, *Streptococcus mutans* and *Candida albicans* play a crucial role by forming biofilms and demineralizing teeth, ultimately leading to the development of dental caries (Kim et al., 2021). Biofilms serve as a vital defense mechanism for these microbes, allowing them to withstand antimicrobial treatments (Zhou et al., 2015). Microbial adherence to tooth surfaces produces extracellular polymeric substances (EPS) that provide protection against antimicrobial agents (Rather et al., 2021). While antimicrobial mouthwash is commonly used to prevent biofilm thickening by inhibiting microbial adhesion during its formation, it may inadvertently contribute to the emergence of resistant strains when used on mature biofilms (Takenaka et al., 2022).

In light of these challenges, alternative strategies that are both effective and environmentally sustainable are urgently needed. One promising approach involves the use of eco-enzymes—bioactive liquids produced through the fermentation of organic kitchen waste, including fruit and vegetable scraps (Gu et al., 2021). These eco-enzymes contain hydrolytic enzymes such as proteases, amylases, and lipases, which can degrade biofilm matrices by targeting their protein and polysaccharide components (Soleha et al., 2023) (Shree et al., 2023). Furthermore, the acidic environment generated during fermentation enhances their antimicrobial activity (Gu et al., 2021). Research by Lestariningsih & Moordiani (2022) demonstrated that an eco-enzyme formulated as a mouthwash can inhibit the growth of *Candida albicans*, achieving a zone of inhibition of 4.83±0.00 mm. Another study by Welfalini et al. (2022) found that eco-enzymes inhibited the growth of *Streptococcus spp*. taken from the ectodermal tissue of a dog's skin, with a zone of inhibition of 8.30±0.25 mm. Controlling the growth of *Streptococcus mutans* and *Candida albicans* can effectively reduce biofilm formation.

The production of eco-enzymes from household waste, such as pineapple cores and lemon peels, not only offers a bioactive alternative for biofilm control but also aligns with sustainable waste management practices. Fruit waste represents a significant portion of domestic organic refuse (Athia et al., 2021). In Indonesia, per capita consumption of pineapple increased from 0.209 kg in 2013 to 0.469 kg in 2017, while lemon consumption reached 12.57 grams per person per day in 2021 (Badan Pusat Statistik, 2021; Fauzi et al., 2021). Globally, lemon consumption in Western Europe also rose from 1.6 kg to 1.9 kg per person between 2013 and 2017 (Confederation of British Industry, 2023). This increase in consumption results in significant waste accumulated in landfills, posing environmental challenges (Aili Hamzah et al., 2021). Left untreated, carbohydrates and proteins from this waste can produce unpleasant odors (Asendy et al., 2018).

Addressing this issue supports the achievement of the United Nations Sustainable Development Goal (SDG) 12, which emphasizes responsible consumption and production

(BAPPENAS, 2023). The dual benefit of eco-enzymes, as biofilm inhibitors and as a means of valorizing food waste, presents a sustainable and innovative solution in the development of natural antimicrobial agents. Maximizing community-level eco-enzyme production may contribute not only to oral health improvement but also to environmental sustainability and antimicrobial stewardship.

METHODS

Tools and Materials

The laboratory instruments and equipment employed in this study included closed plastic containers, universal pH indicators, an incubator, a vortex mixer, micropipette with sterile pipette tips, inoculating loops, 96-well flat-bottom microplates, and a microplate reader. Reagents and materials utilized comprised pineapple core, lemon peel, molasses, distilled water, chlorhexidine (0.2%), Mayer's reagent, Dragendorff's reagent, Bouchardat's reagent, Liebermann-Burchard's reagent, magnesium powder (Mg), hydrochloric acid (HCl), ferric chloride ($FeCl_3$), n-hexane, Benedict's reagent, Lugol's iodine solution, Mueller Hinton Agar (MHA, Himedia®), Mueller Hinton Broth (MHB, Himedia®), glucose, sodium chloride (NaCl, Merck®), McFarland standard 0.5 (Himedia®), crystal violet solution, and ethanol (Merck®). Microbial strains of *Streptococcus mutans* and *Candida albicans* were obtained from the Research Centre for Pharmaceutical Ingredients and Traditional Medicine.

Preparation of Eco-Enzymes

Three eco-enzymes were prepared from pineapple core, lemon peel, and a 1:1 combination of both, using a fermentation process at the Pharmacology Laboratory, UPN Veteran Jakarta. Fresh pineapple cores and lemon peels were collected from a citrus juice vendor at the Angke Fruit Market, West Jakarta, and subsequently washed under running water, cut into small pieces, and weighed. Each organic material was combined with molasses and distilled water in a ratio of 1:3:10. The mixture was placed in closed plastic containers, shielded from direct sunlight, and allowed to ferment at an ambient temperature for a period of three months. After fermentation, the resulting eco-enzyme liquid was filtered to separate it from the residual solids (Gumilar, 2023). Three variants were produced: (1) pineapple core eco-enzyme, (2) lemon peel eco-enzyme, and (3) a 1:1 combination of both.

Phytochemical Screening

Qualitative phytochemical screening was conducted to detect the presence of major secondary metabolites in each eco-enzyme variant. Standard procedures were employed to test for alkaloids, flavonoids, tannins/polyphenols, saponins, steroids/triterpenoids, and carbohydrates using appropriate reagents.

pH Determination

The universal pH indicator was employed to measure the pH of pineapple core, lemon peel, and a combination of both eco-enzymes.

Antimicrobial Activity Test

Minimum Inhibitory Concentration (MIC) Determination

We conducted the MIC determination using the broth microdilution method, following the guidelines of Weinstein et al., 2020. Microbial suspensions were prepared to match a 0.5 McFarland standard and diluted in MHB to obtain a final concentration of 5×10^5 CFU/mL.

MIC determination was performed using the microdilution method with a 96-well flat-bottom microplate, which has twelve columns and eight rows. The first column (A1-H1) served as the negative control, filled with 100 µl of MHB and 100 µl of the bacterial

suspension. The second column (A2-H2) acted as the positive control, containing 100 µl of 0.2% chlorhexidine and 100 µl of the bacterial suspension. The empty wells in rows A to C, with the exception of column 12, were filled with 100 µl MHB. In well A12, 200 µl of pineapple core eco-enzyme was added, and 100 µl of this solution was transferred to well A11. This step was repeated down to well A3, which received a 1/512 dilution of the solution from well A12. The same procedure was followed for the lemon peel eco-enzyme in row B and the 1:1 ratio combination of eco-enzymes in row C. After filling the wells, each well was supplemented with 100 µl of bacterial suspension, resulting in a total volume of 200 µl per well. The microplate was incubated at 35±2°C for 18-24 hours for *Streptococcus mutans* and at 37°C for 24-48 hours for *Candida albicans*. The MIC was identified as the lowest concentration in which the wells remained non-turbid, indicating no microbial growth (Marak & Dhanashree, 2018; Sukandar et al., 2014).

Minimum Bactericidal and Fungicidal Concentration (MBC and MFC) Determination

For each non-turbid well, 5 µl of the content was transferred to a petri dish containing MHA and inoculated with an inoculating loop. The petri dishes were subsequently incubated at 35±2°C for 18-24 hours. The MBC and MFC values were defined as the lowest concentrations of each sample that demonstrated no microbial growth (Afroz et al., 2020; Hendiani et al., 2020; Sukandar et al., 2014).

Antibiofilm Activity Assay

Biofilm Formation Assay

Biofilm formation of *Streptococcus mutans* and *Candida albicans* was done using the microtiter plate method. A total of 100 µL of microbial suspension was mixed with 100 µL of MHB supplemented with 2% glucose in each well. Microplate incubated at 37°C for 24 hours for *Streptococcus mutans* and 48 hours for *Candida albicans*.

Wells were then gently washed with sterile distilled water, stained with 0.1% crystal violet for 15 minutes, washed again, and dried at room temperature. The presence of violet-stained biofilms was confirmed visually. Each well was then solubilised with 200 µL ethanol, and the absorbance was measured at 595 nm. Biofilm formation was classified based on OD595 results that were interpreted by range of specific OD595 values as follows: >0.5 (strong), 0.3–0.5 (moderate), <0.3 (weak), <0.15 (very weak), and 0 (non-adherent) (Al-Bayati & Samarasinghe, 2022; Arjuna et al., 2018; Gunardi et al., 2022; Hejazinia et al., 2020; Neglo et al., 2022; O'Toole, 2011).

Biofilm Growth Inhibition Activity (Antibiofilm Assay)

Antibiofilm activity testing of eco-enzymes was done using the same biofilm formation procedure, with the addition of eco-enzyme treatments. Each eco-enzyme variant was tested at concentrations of 100%, 50%, 25%, 12.5%, and 6.25%. Diluted microbial suspensions of as much as 100 µL were added to 100 µL of MHB supplemented with 2% glucose and considered a negative control in this experiment. The positive control used was 200 µl chlorhexidine 2%, then 100 µl was transferred to the adjacent well, which already contained 100 µl MHB supplemented with 2% glucose. Dilution was repeated until the fifth well with a concentration of chlorhexidine of 0,125%. Each well was then added with 100 µl diluted microbial suspension. Each eco-enzyme sample of 100 µl was added to the well, and then 100 µl microbial suspension was added. Microplates were incubated at 37°C for 24 jam for *Streptococcus mutans* and 48 jam for *Candida albicans*. The incubated microplate was then washed in a sterile aquadest. Each well in the microplate was stained with 200 µl of 0,1% crystal violet for 15 minutes, then washed with sterile distilled water and dried at room temperature. A violet stain inside the well showed biofilm formation. After biofilm formation

was confirmed, each well was reconstituted with 200 µl of ethanol, and its absorbance value (Optical Density or OD) was measured at 595 nm. Experiments were done in triplicate, and then the average and standard deviation values were calculated. Lastly, after the OD value was obtained, the inhibition rate was calculated using these formulas (Alenazy, 2023; Marak & Dhanashree, 2018; Shinde et al., 2021).

$$\text{Inhibition Rate (\%)} = [(OD_{control} - OD_{Treatment})/OD_{control}] \times 100$$

Data Analysis

All experimental procedures were performed in triplicate. The inhibition rates for each concentration and eco-enzyme variant were plotted against the logarithmic concentration to generate regression curves and determine the IC_{50} values, which then was averaged and counted as its standard deviation. Data homogeneity was assessed using Levene's test (p > 0.05 indicating homogeneity), and normality was tested using the Shapiro–Wilk test (p > 0.05 indicating normal distribution) due to a sample size <50 (Usmadi, 2020) (Quraisy, 2022). Statistical significance was evaluated using one-way ANOVA followed by post-hoc Tukey's test for multiple comparisons. Analyses were conducted using IBM SPSS Statistics software, with significance set at $\alpha = 0.05$ (Shinde et al., 2021).

RESULTS AND DISCUSSION

Eco-Enzyme Production

Three variations of eco-enzyme were produced based on the type of organic material used, each yielding 3 liters of final product. A white to yellowish surface film developed during fermentation, identified as a pitera layer, a fungal biofilm rich in amino acids, peptides, proteins, organic acids, vitamins, and minerals (Natasya et al., 2023). Lactic acid bacteria were reported to be found in eco-enzymes. During fermentation, carbohydrates present in the organic materials are first broken down into glucose, which is then converted into pyruvic acid. Under aerobic conditions, pyruvic acid is further transformed into acetaldehyde by pyruvate decarboxylase. Acetaldehyde can either be reduced to ethanol by alcohol dehydrogenase or oxidised into acetic acid via acetaldehyde dehydrogenase (Rukmini & Astuti Herawati, 2023) (He et al., 2022). Concurrently, lactic acid is produced by lactic acid bacteria through lactate dehydrogenase (Wang et al., 2021). Additionally, organic acids derived from the feedstock are bioconverted into enzymes such as protease, lipase, and amylase during fermentation (Gumilar, 2023).

Phytochemical Screening

All three eco-enzyme samples demonstrated similar phytochemical profiles (**Table 1**), containing alkaloids, flavonoids, tannins/polyphenols, saponins, and carbohydrates. Steroid in pineapple eco-enzyme was previously detected by research conducted by (Sai et al. (2023); the results aligned with the steroid compound contained in pineapple. However, in this research, steroids were not detected despite the organic materials used containing steroids. The absence of steroids could be attributed to microbial biotransformation through processes such as isomerization, hydroxylation, oxidation, and condensation (Cano-Flores et al., 2020), which may have degraded the original steroidal compounds.

pH Measurement

The pH values of the eco-enzyme samples (**Table 2**) ranged between 4 and 7, consistent with previous studies on fermented eco-enzymes. The increase in pH level was

expected because of the ammonification process of enzymes and protein contained in eco-enzymes. Protein, with the help of microbes, undergoes decomposition into amino acids, its most simple form, and subsequently deaminated, resulting in the production of ammonia, a base that increases the pH of eco-enzyme (Pashaei et al., 2022). This process also altered the odor of eco-enzymes, particularly those made with mixed organic materials, resulting in a strong and unpleasant smell. These changes indicate potential degradation in eco-enzyme quality, including reduced antimicrobial activity.

Table 1. Qualitative Phytochemical Screening Results of Eco-Enzyme

Phytochemical Compound	Organic Matters of Eco-Enzyme			Description
	Pineapple Core	Lemon Peel	Combination 1:1	
Alkaloid				
- Mayer	-	-	-	No precipitate formed
- Dragendorff	+	+	+	Precipitate formed
- Bouchardat	+	+	+	Precipitate formed
Flavonoid	+	+	+	Orange color change
Tanin/polyphenol	+	+	+	Blackish color change
Saponin	+	+	+	Stable bubble formed
Steroid/ triterpenoid	-	-	-	No color change
Carbohydrate				
- Benedict	+	+	+	Precipitate formed
- Lugol	-	-	-	No color change

Table 2. pH Determination of Eco-Enzyme Sample

Sample	pH
Pineapple Core Eco-Enzyme	4 – 5
Lemon Peel Eco-Enzyme	4 – 5
Combination 1:1 Eco-enzyme	6 – 7

Antimicrobial Activity

The MIC, MBC, and MFC values of the eco-enzymes and chlorhexidine against *Streptococcus mutans* and *Candida albicans* are shown in **Table 3**. The antimicrobial activity of eco-enzymes was suspected because of the presence of acetic acid in them (Vidalia et al., 2023). Based on previous research by Salma and Ratni in 2022, the acetic acid content of eco-enzymes that fermented for 90 days was as much as 9.69%. Acetic acid can interfere with the structure of the microbial cell wall, resulting in a loss of energy (in the form of ATP) for the microbe. The acetic acid contained in the eco-enzyme was enough to inhibit the growth of *Candida albicans*, which requires an acetic acid concentration of 10% to inhibit its growth (Zinn & Bockmühl, 2020). That concentration was also enough to kill *Streptococcus mutans* entirely because it only needed 3% acetic acid with an exposure time of 30 minutes (Halstead et al., 2015).

Antibiofilm Activity

The antibiofilm activity of the three eco-enzyme samples was evaluated against *Streptococcus mutans* and *Candida albicans*, with appropriate controls included. The concentrations tested were selected based on MIC results, under the assumption that

concentrations capable of inhibiting microbial growth might also impede biofilm formation (**Table 4**). Biofilm development is a complex process regulated by quorum sensing, a mechanism that facilitates intercellular communication among microbial cells. This signalling system coordinates various physiological activities, including the transition from planktonic to biofilm-associated lifestyles, thereby enhancing microbial survival and virulence (Preda & Săndulescu, 2019). Quorum sensing mechanism produces protease, an extracellular enzyme that plays a crucial role in biofilm maturation and dispersion (Dewatisari et al., 2023). Protease produced from quorum sensing hydrolyzes biofilm structure, resulting in the dispersion of microbe cells from the biofilm, leading to the spread of microbes (Ramírez-Larrota & Eckhard, 2022).

Table 3. Minimum inhibitory value and minimum bactericidal/fungicidal concentration of eco-enzyme and chlorhexidine against *Streptococcus mutans* and *Candida albicans*

Sample	MIC Value ± Standard Deviation		MBC and MFC Value ± Standard Deviation	
	Streptococcus mutans	*Candida albicans*	*Streptococcus mutans*	*Candida albicans*
Chlorhexidine	≤ 0.00625% ± 0%	≤ 0.00625% ± 0%	≤ 0.00625% ± 0%	≤ 0.00625% ± 0%
Pineapple Core Eco-Enzyme	25% ± 0%	50% ± 0%	50% ± 0%	No inhibition
Lemon Peel Eco-Enzyme	50% ± 0%	50% ± 0%	No inhibition	No inhibition
Combination 1:1 Eco-enzyme	50% ± 0%	50% ± 0%	No inhibition	No inhibition

Table 4. Results of Antibiofilm Activity Testing of Eco-Enzyme and Chlorhexidine Samples

Sample	IC_{50} Value ± Standard Deviation	
	Streptococcus mutans	*Candida albicans*
Chlorhexidine	2936.28 ± 1756.30 ppm	1865.69 ± 433.59 ppm
Pineapple core eco-enzyme	84229.72 ± 68909.47 ppm	145026.38 ± 56157.65 ppm
Lemon peel eco-enzyme	185613.82 ± 66102.54 ppm	557162.94 ± 252732.39 ppm
Combination 1:1 eco-enzyme	68526.23 ± 54994.44 ppm	2235.30 ± 3122.52 ppm

Eco-enzymes are known to contain protease enzyme, which may contribute to destroy and prevent the forming of a biofilm matrix (Soleha et al., 2023). Referring to the organic material used for the production of the eco-enzyme, specifically, eco-enzymes derived from pineapple core are likely to retain bromelain, a proteolytic enzyme naturally present in pineapple, even after fermentation (Ilyas, 2020). The IC50 values for the antibiofilm activity of eco-enzyme with single organic materials shows that pineapple core eco-enzyme was more active than lemon peel eco-enzyme, likely due to bromelain's dual role in degrading the microbial cell wall and inhibiting biofilm formation (Muhsinin et al., 2021). Furthermore, the eco-enzyme made with mixed organic materials demonstrated the most effective antibiofilm activity, evidenced by the lowest IC_{50} values. This benefit could be attributed to its higher pH, which aligns with the optimal activity range of protease enzymes (~pH 6) (Soleha et al., 2023). Optimal protease activity contributes significantly to microbial cell death and prevents biofilm development (Liaqat et al., 2021). The theory was in line with the research results, where the eco-enzyme with the highest pH value, eco-enzyme with The combination

of organic materials exhibited better antibiofilm activity against both *Streptococcus mutans* and *Candida albicans*.

CONCLUSION

This study demonstrated that all three eco-enzymes from the pineapple core, lemon peel, and organic material combinations have antimicrobial activity against *Candida albicans* with an MIC value of 50% ± 0% and *Streptococcus mutans* with an MIC value of 50% ± 0%, except for the pineapple core eco-enzyme, which got an MIC value of 25% ± 0% and an MBC value of 50% ± 0%. All three eco-enzymes were beneficial for inhibiting biofilm formation, as seen from the one-way ANOVA statistical significance of 0.023 for *Streptococcus mutans* and 0.002 for *Candida albicans*. The effective concentrations of pineapple core, lemon peel, and organic material combination eco-enzyme that inhibit 50% of *Streptococcus mutans* growth are 84229.72 ± 68909.47 ppm, 185613.82 ± 66102.54 ppm, and 68526.23 ± 54994.44 ppm. At the same time, for *Candida albicans*, they are 145026.38 + 56157.65 ppm, 557162.94 ± 252732.39 ppm, and 2235.30 ± 3122.52 ppm.

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AUTHOR CONTRIBUTION

HA: literature search; experimental studies; data analysis; manuscript preparation.
EPR: Concepts or ideas; design; definition of intellectual content; manuscript editing; manuscript review.
RR: Design; manuscript editing; manuscript review.
DLCP: Manuscript review.
AWS: Manuscript editing; manuscript review.

CONFLICT OF INTEREST

None to declare

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