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Eco-enzyme Characterization of Fruit Peel Waste Mixture and Test of Antibacterial Activity against Bacteria Causing Dental Caries

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Abstract

Eco-enzyme is a product of facultative aerobic fermentation for 3 months from organic fruit and vegetable waste. In this study, eco-enzyme was made from a mixture of fruit peel waste (papaya, orange and pineapple) with the addition of 6 L of water. Eco-enzyme products were characterized by organoleptic properties, chemical composition, while enzyme activity and antibacterial activity tests against *Lactobacillus acidophilus* and *Streptococcus mutans* bacteria that cause dental caries. The results show that the eco-enzyme product had a dominant orange peel sour flavor, was cloudy brown in color with a volume of 8.4 L from 6 L of added water. After fermentation of the eco-enzyme, the pH value was 3.1. The characterization results depict that eco-enzyme contained acetic acid and lactic acid with a total acid content of 2.26%, 40.45 mg/mL protein, and secondary metabolites in the form of alkaloids, flavonoids, and saponins. The activity of several enzymes from eco-enzyme was protease 0.0246 U/mL, amylase 0.0032 U/mL and lipase 5 U/mL. Eco-enzyme concentration 80 (%v/v) inhibits the antibacterial activity of *L. acidophilus* with a strong inhibition zone response, eco-enzyme concentration 40% (v/v) has a moderate inhibition zone response with inhibition zone diameters of 23.33 mm and 8.23 mm respectively. The minimum inhibitory concentration (MIC) and minimum killing concentration (MKC) values of eco-enzyme against *S. mutans* and *L. acidophilus* bacteria were 2500 ppm and 5000 ppm respectively. These results demonstrate its promise as a natural antibacterial agent for sustainable waste management and dental health.

Keywords: *eco-enzyme, fruit-peel waste, L. acidophilus, S. mutans, antibacterial*

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Abstrak (Indonesian)

Ekoenzim merupakan produk hasil fermentasi fakultatif aerob selama 3 bulan dari limbah organik buah dan sayur. Pada penelitian ini, ekoenzim dibuat dari campuran limbah kulit buah (papaya, jeruk dan nanas) dengan penambahan 6 L air. Produk ekoenzim dikarakterisasi sifat organoleptik, komposisi kimia, aktivitas enzim dan uji aktivitas antibakteri terhadap bakteri *Lactobacillus acidopilus* dan *Streptococcus mutans* penyebab karies gigi. Hasil penelitian diperoleh produk ekoenzim memiliki aroma asam kulit jeruk dominan, berwarna cokelat keruh dengan volume 8,4 L dari 6 L penambahan air. Setelah fermentasi ekoenzim didapatkan larutan dengan pH 3.1, Hasil karakterisasi menunjukkan ekoenzim mengandung asam asetat dan asam laktat dengan kadar asam total 2,26%, protein 40,45 mg/mL, dan metabolit sekunder berupa alkaloid, flavonoid, dan saponin. Aktivitas beberapa enzim dari ekoenzim adalah protease 0,0246 U/mL, amylase 0,0032 U/mL dan lipase 5 U/mL. Aktivitas antibakteri terhadap bakteri penyebab karies gigi termasuk kategori sangat kuat terhadap *S. mutans* pada konsentrasi 40% (v/v) dan sedang terhadap *L. acidophilus* dengan diameter zona hambat berturut-turut 23,33 mm dan 8,23 mm. nilai Konsentrasi Hambat Minium dan Konsentrasi Bunuh Minimum

ekosenzim terhadap bakteri *S. mutans* adalah 2500 ppm dan *L. acidophilus* 5000 ppm. Hasil ini menunjukkan bahwa ekoenzim dapat bertindak sebagai agen antibakteri alami untuk pengelolaan limbah berkelanjutan dan kesehatan gigi.

Kata Kunci: ekoenzim, limbah kulit buah, L. acidophilus, S. mutans, antibakteri

INTRODUCTION

One of the environmental problems faced by society is waste that is no longer used by its owner. There are two types of waste in general, namely inorganic waste and organic waste. Organic waste is a type of waste that has undergone decomposition which can come from nature and the remains of living things including plants, animals and humans [1]. The waste pile in Indonesia based on data from the National Waste Management Information System (SIPSN) reaches 360.755.442,70 tons/year with 40.5% being food waste which is a type of organic waste composition [2]. Uncontrolled disposal generates serious heavy metals pollution occurring in the water, soil, and plants, organic waste picking within open dump sites pose to serious health risk people working on these areas [3], release of organic waste in water bodies improve the marine litter globally, enhancing environmental contamination [4]. Therefore, organic waste mismanagement is cause of sever and various environmental and social impacts, which do not allow improvements in sustainable development.

One way to utilize organic waste is to process it into eco-enzymes. Eco-enzymes are complex liquids consisting of protein chains (enzymes), organic acids, and mineral salts which are made by carrying out a fermentation process from various kitchen wastes such as fruit and vegetable peels [5]. Eco-enzymes contain organic acids in the form of lactic acid and acetic acid which are useful in inhibiting bacterial growth [6]. Organic acids in eco-enzymes come from metabolic processes that are naturally produced from basic eco-enzyme ingredients, including vegetables and fruits. Organic acids can inhibit transmembrane potential and substrate transport as well as make the structure of pathogenic bacterial cells, one of which is the cytoplasm, become acidic [7]. Eco-enzyme has various benefits in the health sector, including as mouthwash, sterilization fluid, and replacement fluid for people who are allergic to soap [8]. One of the uses of eco-enzymes that has been carried out by [9] which shows that eco-enzyme solution from pineapple skin at a concentration of 50% can inhibit the growth of *Staphylococcus aureus* and *Propionibacterium acnes* bacteria that can infect the skin.

Eco-enzymes can also be used as a mouthwash that can help control dental plaque that causes caries. Dental caries is the demineralization of hard tooth tissue due to acids produced by acidogenic and aciduric species in the mouth. An important component that causes caries is dental plaque which can be removed chemically using mouthwash. Mouthwash can help control dental plaque because it can reach areas that are difficult to reach with a toothbrush. Currently, mouthwashes are available with various active ingredients, one of which is a chemical in the form of sodium hypochlorite (NaOCl) which has been reported to have antibacterial effects against cariogenic bacteria in dentinal tubules and antiplaque effects [10]. NaOCl has several disadvantages because it is a very strong oxidizing solution and corrosive. This result in irritating surrounds tissue at high concentrations [11].

A potential substitute for sodium hypochlorite as a mouthwash is needed due to reason mentioned above. One of which is from natural plant extracts in the form of fruit peels processing fermentation. The antibacterial property of the fruit peels increases because organic substances decompose and produce secondary metabolites. Papaya peels contain papain which has a less harmful effect on vital tissue compared to NaOCl since its proteolytic activity selectively targets unhealthy tissue. In addition, papaya peels have potential anti-inflammatory effects minimizing chronic inflammatory processes and tissue damage. Pineapple and orange peels have been shown to have antimicrobial and anti-inflammatory properties. The high content of polyphenol and flavonoid compounds in pineapple and orange extracts has excellent antimicrobial and antioxidant activity. Bromelain from pineapple extract is effective in killing bacteria by disrupting the peptidoglycan and polysaccharide components of bacterial cell membranes [12]. Previous study concluded that orange and pineapple fruits can be used as a antimicrobial agents for the protection from selected plant and animal pathogen [13]. Research from Mavani, *et al.* (2020) [6] also revealed that the 100% natural pineapple-orange peel eco-enzyme (M-EE) and 50% papaya peel eco-enzyme (P-EE) as well as 2.5% NaOCl were no significant difference in antimicrobial against *Enterococcus faecalis*. Nevertheless, utilization of eco-enzyme mouthwash

from a mixture of papaya peel, orange peel and pineapple peel on the antibacterial activity of *Lactobacillus acidophilus* and *Streptococcus mutans* which cause dental caries. The utilization of eco-enzyme mouthwash from a mixture of papaya peel, orange peel and pineapple peel on the antibacterial activity of *Lactobacillus acidophilus* and *Streptococcus mutans* which cause dental caries has a lack literature. Eco-enzyme's potential as a natural antimicrobial agent against oral bacteria, particularly in disrupting biofilms formed by species like *Streptococcus mutans* and *Streptococcus mutans*. Its ability to eradicate multi-species biofilms through mechanisms involving secondary metabolites, organic acids, and enzymatic activity highlights its relevance in addressing oral microbiota dysbiosis. This contribution underscores eco-enzyme's role as an environmentally friendly alternative for oral caries, paving the way for sustainable and safer solutions in dental health management. Therefore, this research is urgent to be carried out so that it will contribute to another knowledge regarding eco-enzymes.

MATERIALS AND METHODS

Materials and Instruments

The instruments used in this study include Incubators, laminar air flow, vortex mixers, Ohaus analytical scales, Orion Aquamate 8000 UV-Vis spectrophotometers, and various laboratory glass wares. The materials used in this study include orange peel, papaya peel, pineapple peel, brown sugar, water, isolates of *L. acidophilus* FNCC 0051 and *S. mutans* ATCC 25175 bacteria from Food and Nutrition Culture Collection, Gajah Mada University.

Procedure

Eco-enzyme making

A total of 600 grams of papaya peel, 600 grams of orange peel, and 600 grams of pineapple peel were put into a gallon, added with 600 grams of brown sugar and 6 liters of water in a ratio of 3:1:10 then stirred until evenly mixed. The gallon containing the mixture was tightly closed, then fermented for three months. In the first month, once a day, the gallon cap was opened to release the fermentation gas. After fermentation for three months, the eco-enzyme was opened, and then the eco-enzyme was filtered using a sieve lined with a clean cloth to separate the eco-enzyme and fruit peel residue. After that, the volume obtained was measured. Then, the eco-enzyme was filtered again using Whatman No. 1 paper for further testing [14].

Organoleptic test

The filtered eco-enzymes were tested organoleptically by distributing questionnaires to 30 panelists who observed the eco-enzymes in the form of flavor, color, and viscosity. [15]

Determination of Chemical Composition

Identification of acetic acid and lactic acid

The filtered eco-enzyme at 0.1 mL was put into a test tube. Sodium bicarbonate of 0.05 M with the total 1 mL was added. Following this, a positive acetic acid result was indicated by the formation of gas bubbles. A total of 1 mL of the filtered eco-enzyme was put into a test tube. Next, 1 mL of 1% iodine and 1 mL of 0.1 N sodium hydroxide was added, and then a positive lactic acid result was indicated by a brownish yellow color.

Determination of total acid content

The determination of total acid content in eco-enzyme was done using the alkalimetric titration method with sodium hydroxide solution as a titer. Standardization was carried out by using 0.1 N oxalic acid. A total of 10 mL of oxalic acid was put into an Erlenmeyer flask, 3 drops of pp indicator were added, and titration was carried out until the solution turned pink. The volume of sodium hydroxide used was recorded, and the normality of sodium hydroxide was calculated. The normality of sodium hydroxide obtained was 0.15 N. A total of 10 mL of the filtered eco-enzyme was pipetted and put into an Erlenmeyer flask. Phenolphthalein indicator at 0.3 mL were added so titration was carried out until the solution changed color, and the volume of sodium hydroxide used was recorded. The total acid content in the eco-enzyme was calculated. Then, eco-enzyme solution was measured by pH meter.

Determination of protein content by Biuret method

Filtered eco-enzyme with a total of 0,6 mL was taken, put into a test tube and 6 mL of Biuret reagent was added. The mixture was stirred using a vortex mixer until homogeneous and left at room temperature for 10 minutes. The absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 597 nm and the protein content of the eco-enzyme was calculated [16].

Phytochemical Screening

Alkaloids test

In determining alkaloids, 1 mL of eco-enzyme was put into a test tube; 1 mL of chloroform was added. A total of 3 drops 10% ammonia and 10 drops of 95% H_2SO_4 were added. Then the mixture was homogenized and left until two phases were formed. The upper phase was pipetted and put into 3 different

test tubes. Each tube was added with 3 drops of different reagents including Mayer's reagent (mercuric chloride, potassium iodide, and distilled water), Dragendorff's reagent (potassium iodide, bismuth subnitrate, acetic acid and distilled water) and Bouchardat's reagent (potassium iodide, iodine, distilled water). The results are positive if there is a white precipitate after the addition of Mayer's reagent, changing to be orange precipitate after the addition of Dragendorff's reagent, and becoming a brown precipitate after the addition of Bouchardat's reagent.

Flavonoids test

In the determination of flavonoids, 1 mL of eco-enzyme was put into a test tube, 2 mg of magnesium powder (Mg) and 3 drops of 37% HCl were added. The result is positive if the color changes to a red, orange or yellow color.

Saponin test

In the determination of saponins, 1 mL of eco-enzyme was put into a test tube, 10 mL of hot water was added, cooled, and shaken vigorously for 10 seconds. The result is positive if it produces foam as high as 1-10 cm for no less than 10 minutes.

Tanin test

In the determination of tannins, 1 mL of eco-enzyme was put into a test tube and 5 drops of 1% $FeCl_3$ was added. The result is positive for catechol tannins if they are blue-green and positive for pyrogallol tannins if they are blue-black in color.

Terpenoids and steroids test

In the determination of terpenoids and steroids, 2 mL of eco-enzyme was put into a test tube and 2 drops of Libermann-Burchard reagent (acetic anhydride and concentrated sulfuric acid) were added. Positive terpenoid and steroid results if a brownish or violet ring and blue ring are formed at the border of the solution respectively [17].

Enzyme Activity Test

Determination of protease enzyme activity

A total of 0.5 mL of enzyme solution was put into a test tube. 1 mL of 0.05 M phosphate buffer solution pH 7 was added, and then 1% skim milk was added. Following this, the mixture was incubated in a water bath at 30 °C for 15 minutes. The reaction was stopped by adding 1 mL of 0.4 M trichloroacetic acid (TCA). The solution was then centrifuged to obtain the supernatant. A total of 0.5 mL of supernatant was taken and added with 2.5 mL of 0.5 M Na_2CO_3, incubated for 10 minutes at room temperature, then 0.5 mL of Folin-Ciocalteu reagent (water, sodium tungstate, sodium molybdate, phosphoric acid, hydrochloric acid, lithium sulfate, and bromine) which had been added with water in a ratio of (1:2) and re-incubated for 30 minutes at room temperature. The incubation results were measured with a spectrophotometer at a wavelength of 660 nm [18]. The absorbance results were converted from tyrosine.

Determination of lipase enzyme activity

A total of 2 grams of palm cooking oil was weighed in a 150 mL Erlenmeyer flask. 1 mL of filtered eco-enzyme and 4 mL of 0.05 M phosphate buffer solution, pH 7.5 were added, stirred with a magnetic stirrer for 1 hour. Next, 10 mL of 96% acetone-ethanol (1:1) was added and stirred until homogeneous. 2-3 drops of PP indicator were added to the stirred solution, titrated with 0.5 N KOH in 0.5 N alcohol. Titration was stopped when the color of the solution turned pink and did not disappear, and then the titration volume was noted. The blank solution was made in the same way as the sample, but 96% acetone-ethanol solution (1:1) was added before stirring to deactivate the enzyme. Then the lipase enzyme activity of eco-enzyme was calculated.

$$\text{Lipase Activity } (IU/mL) = \frac{(A-B)x1000xI\ (NaOH)}{W\ (g)x\ t} \quad (1)$$

A = ml NaOH for sample titration
B = ml NaOH for blank titration
N = Normality of NAOH
1000 = conversion of mmol to µmol
W = weigh of sample (g)
t = incubation time (minutes)

Determination of amylase enzyme activity

The amylase enzyme activity was tested using the DNS method and expressed in international units (U/mL). A total of 1 mL of eco-enzyme was put into a test tube. 1 mL of 1% starch substrate solution in 50 mM sodium phosphate buffer pH 7 was added. The mixture was incubated for 10 minutes at 50 °C, 2 mL of DNS reagent (3,5-dinitrosalicylic acid, Sodium hydroxide, distilled water, potassium sodium tartrate) was added and boiled for 10 minutes in a water bath at 100°C, and cooled. Then the absorbance was measured using a UV-Vis spectrophotometer at a scanning wavelength (λ) of 540 nm and the amylase activity of eco-enzyme was calculate

$$UA = \frac{(Asp-Abl)}{(Ast-Abl)}\ x\ FP\ x\ \frac{1}{T} \quad (2)$$

UA = activity unit (IU/ml)
Asp = sample absorbance
Ast = standard absorbance
Abl = blank absorbance
FP = dilution factor
t = incubation time (minutes)

Determination of Antibacterial Activity

Determination of clear zone

Diameter The antibacterial activity test was carried out using the agar diffusion method using a 6 mm diameter paper disc with bacteria *L. acidophilus* and *S. mutans*. The activity test was three replicated. The bacteria that will be tested were smeared onto Nutrient Agar media for *S. mutans* bacteria and MRS Agar for *L. acidophilus* bacteria using an L rod, and left for 15 minutes. The paper disc was dipped into eco-enzyme that had been diluted to a concentration of 80, 40, 20, 10% (v/v), positive control (amoxicillin) and negative control (10% DMSO). The paper disc that had been dripped was placed using tweezers on the surface of the media that had been inoculated with bacteria. After that, the media containing the bacteria was incubated for 24 hours for *S. mutans* bacteria and 48 hours for *L. acidophilus* bacteria at a temperature of 37 °C. After incubation, the bacterial growth was observed and the clear zone formed was measured using a ruler or caliper.

Determination of minimum inhibitory concentration

The determination of MIC was carried out using the dilution method or multilevel dilution with a ratio of 1:2 (w/v). A total of 11 sterilized test tubes were prepared. Then each tube was labeled 1-9, tube 10 was labeled K (+) or positive control containing amoxicillin. Tube 11 was labeled K (-) or negative control containing 10% DMSO. Tube 1 was filled with 4 mL of sample that had been diluted to 10,000 ppm. Tubes 2 - 9 were filled with 2 mL of Nutrient broth liquid media for *S. mutans* bacteria and MRS broth for *L. acidophilus* bacteria. Then, 2 mL of solution was taken from tube 1 and put into tube 2, mixed until homogeneous. At the same time, it was done until tube 9 until the extract concentration was obtained with a ratio of 1:2 (w/v). The next step, a bacterial suspension that had been equalized with the McFarland 0.5 standard was added, which was equivalent to an estimated amount of bacterial suspension of 1.5 x 108 CFU/mL. 1 mL was poured into tubes 1-9 and tubes K (+) and K (-). All tubes were then incubated for 1x24 hours for *S. mutans* and 2x24 hours for *L. acidophilus* bacteria at a temperature of 37^{o}C with 3 repetitions.

The incubation was observed using the turbidimetry method or turbidity observation. If the turbidity of the tube is still equivalent to or more turbid than the K (+) tube, it can be said that the bacteria can still grow well, but if the solution in the tube looks clearer than the K (+) tube, it means that growth is starting to be inhibited. Furthermore, absorbance measurements were carried out using a UV-Vis spectrophotometer at a scanning wavelength of 625 nm which is comparable to an OD value of 1.102. Absorbance measurements were carried out before and after incubation. The testing process was repeated 3 times, so that the MIC value could be determined from the difference in absorbance values before and after incubation.

RESULTS AND DISCUSSION

Eco-enzyme Product

The resulting eco-enzyme was depicted in **Figure 1**. The eco-enzyme was a stable brown liquid, with a dominant sour flavor of orange peel, and has a volume of 8.4 liters from 6 liters of water added during the manufacture of eco-enzyme.

Figure 1. Eco-enzyme Fermentation Results

The sour aroma of eco-enzyme comes from the work of facultative anaerobic bacteria that produce acid. The color produced from the eco-enzyme solution varies between light brown to dark brown depending on the type of sugar used and the length of fermentation time. The increase in volume after fermentation is due to the water content of the fruit skin waste used.

Organoleptic

The level of preference in the organoleptic test consists of 5 choices, namely Dislike Strongly (STS) = 1, Dislike (TS) = 2, Somewhat Like (AS) = 3, Like (S) = 4, Very Like (SS) = 5.

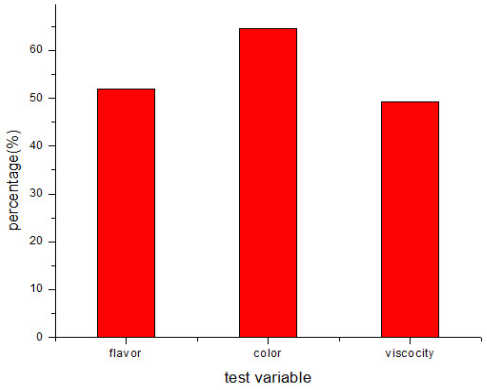

Figure 2. Organoleptic test result

Based on **Figure 2**, it shows that the organoleptic test consists of flavor, color and viscosity levels of eco-enzyme with percentage parameters where very dislike is given a value of 1, dislike is given 2, somewhat like is given 3, like is given 4, very like is given 5, and then the results obtained are divided by the total panelists. The result of preference level for the flavor of the eco-enzyme was 52%, the difference color of the eco-enzyme was 64.6%, and the viscosity of the eco-enzyme was 49.3%, where the panelists' preference level for the color of the eco-enzyme was greater than the aroma and viscosity of the eco-enzyme. This is due to the metabolic activity of microorganisms that result in the breakdown of substrates by bacteria, resulting in changes in the aroma, color, and texture of the fruit skin from eco-enzymes. The conclusion of this study is that the use of fruit skin as a basic ingredient for making eco-enzymes affects the level of respondent preference. Eco-enzymes can be used as natural fertilizers for plant growth and can reduce household waste [19].

Acid content

In this study, after fermentation of the eco-enzyme, the pH value was 3.1. The pH value was influenced by the organic acid content, where the higher the organic acid content, the lower the pH value. This shows that the pH value in this study was low due to the high organic acid content. The differences in pH values in eco-enzyme solutions can be caused by the type of organic material used [20].

Lactic acid undergoes oxidation by iodine ions in the iodine solution, which produces pyruvic acid and the formation of iodide ions. The pyruvic acid formed reacts with excess iodine ions to form a yellow-brown compound, namely 2,3-diiodoosuccinic acid. Sodium hydroxide acts as a base to hydrolyze lactic acid into pyruvic acid. The eco-enzyme product in this study also contains a total acid content of 2.26%. The percentage of titrated acid content in several types of fruits and vegetables will differ due to differences in the number of organic acids contained [21].

The eco-enzyme product in this study was positive for acetic acid and lactic acid, where in the acetic acid content test bubbles were formed when sodium bicarbonate solution was added to the eco-enzyme. Sodium bicarbonate is a weak base that can react with acetic acid and produce carbonic acid. Carbonic acid decomposes into carbon dioxide gas and water. The carbon dioxide gas bubbles that are formed indicate the presence of acetic acid in the eco-enzyme. In the lactic acid content test, a yellow-brown color was formed when iodine and sodium hydroxide were added to the eco-enzyme [22].

Protein content

The protein content of eco-enzyme in this study was obtained at 4.045 mg/mL. Protein content is influenced by fermentation time and the type of extract measured. The protein content value will decrease over fermentation time. This is because there is a process of degradation of organic matter by microorganisms contained in the eco-enzyme solution. Protein content is also influenced by the type of extract that is reduced where the protein content of the purification results will be smaller than the protein content of the crude extract since the crude extract solution of the enzyme produced still contains many types of protein [23].

Phytochemical Screening

The compound groups found in the mixed eco-enzyme of pineapple, papaya, and orange peel waste in this study are presented in **Table** 1. The presence of Alkaloids is indicated by the formation of a white precipitate after the addition of Mayer's reagent. The precipitate formed is a potassium alkaloid complex, the presence of nitrogen atoms in alkaloid compounds that have free electron pairs can be used to form coordinate covalent bonds with metal ions. Nitrogen in alkaloids will react with the metal ion K^+ from potassium tetraiodomercurate (II) to form a potassium-alkaloid complex [24].

Table 1. Phytochemical Screening

Compounds	Result
Alkaloid	+
Flavonoid	+
Saponin	+
Tanin	-
Terpenoid dan steroid	-

Description: (+) contains the tested compound group
(-) does not contain the tested compound group

The presence of an orange or light brown precipitate after the addition of Dragendorff's reagent also indicates the presence of alkaloids. The precipitate is a potassium alkaloid. Nitrogen in alkaloids with Dragendorff's reagent is used to form coordinate covalent bonds with K^+ which is a metal ion. Brown precipitate after adding Boucharadat's reagent also indicates the presence of alkaloids. The precipitate formed occurs due to the presence of coordinate covalent bonds between the metal ion K^+ and the alkaloid so that a potassium-alkaloid complex is formed which precipitates [25].

The presence of flavonoid compounds is indicated by the formation of yellow, orange, red colors due to the reduction of the benzopyrone

nucleus in the flavonoid structure by hydrochloric acid and magnesium so that a flavylium salt is formed which is yellow, orange to red. Testing flavonoids with the addition of Mg and HCl functions to reduce the benzopyrone nucleus contained in the flavonoid structure so that a flavylium salt is formed [26]. On the other side, the presence of saponin compounds is indicated by the formation of foam. The foam formed is caused by saponins having hydrophobic and hydrophilic groups which can act as active surfaces in the formation of foam. The emergence of foam indicates the presence of glycosides which have the ability to form foam in water which is hydrolyzed into glucose and other compounds. Compounds that are partially soluble in water (hydrophilic) and compounds that are soluble in nonpolar solvents (hydrophobic) as surfactants in saponins can reduce surface tension. When the compound is shaken, the hydrophilic group will bind to water while the hydrophobic group will bind to air to form foam [27].

Enzyme Activity

Protease, lipase, and amylase enzymes are included in the hydrolase enzyme group. Hydrolase enzymes are enzymes that catalyze the hydrolysis reaction of a substrate or the breakdown of a substrate with the help of water molecules. Protease activity in this study used the Bergmeyer and Grassl method and obtained a protease activity of 0.0246 U/mL. The working principle measures the amino acid tyrosine that is hydrolyzed after being separated from its substrate. The enzyme will break down the casein substrate with the help of water into amino acids and peptides [16].

The substrate used is casein which is a milk protein consisting of phosphoproteins bound to calcium caseinate. Amino acids produced from the hydrolysis of casein by protease are separated from proteins that have not been hydrolyzed using trichloroacetic acid (TCA). In addition, TCA also functions to inactivate protease and stops the protease incubation time. The filtrate and sediment formed are separated by centrifugation. Addition of Na_2Co_3 which can bind water in solution Folin-Ciocelteau was used as a dye reagent that reacts with protein to form a blue color. The principle of the Folin-Ciocalteu method is the oxidation of phenolic hydroxyl groups [28].

In the protease activity test, tyrosine is used to measure protease activity. Lipase activity in this study used the titrimetric method where the lipase enzyme activity was obtained at 5 U/mL. The specific activity of the enzyme is influenced by the protein content, the higher the specific activity of an enzyme, the higher the purity of the enzyme. This is due to the separation of other proteins that are not enzymes. This lipase enzyme can break down ester bonds in fats into fatty acids and glycerol. Lipase is a group of enzymes that generally function in the hydrolysis of triacylglycerols (triglycerides) [29] to produce long-chain fatty acids and glycerol as illustrated in **Figure 3**. The reaction is:

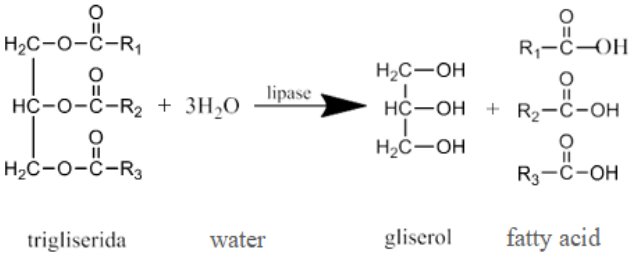

Figure 3. Hydrolysis Reaction [24]

Amylase enzyme activity was measured using the DNS method. In this study, amylase enzyme activity was obtained at 0.0032 U/mL. The principle of the DNS method is based on the reaction process of the aldehyde group in the polysaccharide chain which is oxidized to a carboxyl group, and at the same time, the aldehyde group of sugar will reduce 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid. Reducing sugar will be bound by the DNS solution and as long as there is reducing sugar in the solution being tested, the reaction will continue until the reducing sugar has finished reacting. Amylase enzyme activity is influenced by pH. At optimum pH, the enzyme is at the most appropriate ionization level to bind to the substrate. Changes in pH cause the changes in the charge of amino acid residues, especially those that make up the active center of the enzyme, and the charge of amino acid residues that make up the substrate [20].

Antibacterial Activity

Testing of the antibacterial activity of eco-enzymes was carried out using the disc diffusion method with test bacteria *L. acidophilus* and *S. mutans*. Antibacterial activity test was conducted to determine the effect of eco-enzyme in inhibiting the growth of bacteria that cause dental caries. The results of the antibacterial activity test of eco-enzyme samples are shown in **Figure 4**, while the results of the inhibition zone measurements are shown in **Table 2** bellow.

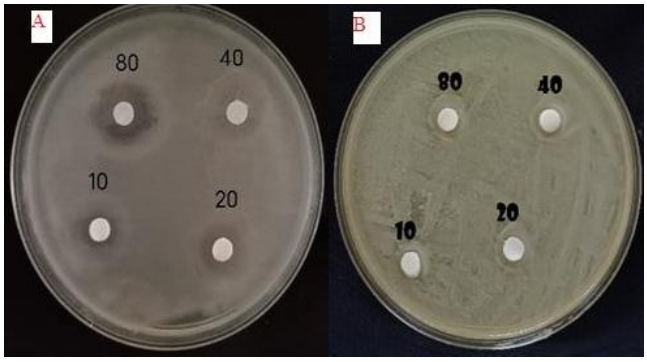

Figure 4. Eco-enzyme antibacterial activity against *S. mutans* (A*), L. acidophilus* (B)

Table 2. Inhibition zone diameter of eco-enzyme

Test Compound	Concentration (%v/v)	Inhibition zone diameter (mm) Mean ± SD	
		L. acidophilus	***S .mutans***
Eco-enzyme	80	11.33±0.65	26.83±2.27
	40	9.06±1.10	23.23±1.01
	20	8.23±0.68	19.13±1.79
	10	7.53±0.75	15.30±0.98
control +	-	34.33±1.52	25.66±0.88
control -	-	6.00±0.57	6.00±0.34

Table 3. Value of difference in optical density (ΔOD)

Concentration (ppm)	Optical Density (OD)	
	L. acidophilus	***S. mutans***
Positive control (Amoxicillin)	-0.002	-0.006
Negative control (DMSO 10%)	0	0
10000	-0.026	-0.038
5000	-0.035	-0.043
2500	0.028	-0.039
1250	0.089	0.029
625	0.377	0.098
312.5	0.388	0.364
156.25	0.544	0.587
78.125	0.729	0.529
39.062	0.969	0.390

Eco-enzyme concentration 80 (%v/v) inhibits the antibacterial activity of *L. acidophilus* with a strong inhibition zone response, eco-enzyme concentration 40; 20; 10% (v/v) has a moderate inhibition zone response. Eco-enzyme concentration 80; 40% (v/v) inhibits the antibacterial activity of *S. mutans* with a very strong inhibition zone response, eco-enzyme concentration 20; 10% (v/v) has a strong inhibition zone response.

Based on the calculation of the percentage of sample inhibition, it shows that eco-enzyme at all concentrations inhibits the antibacterial activity *of L. acidophilus* with a weak inhibition zone response, while eco-enzyme at a concentration of 80; 40; 20% (v/v) inhibiting the antibacterial activity of *S. mutans* has a strong response, concentration 10% (v/v) has a moderate response. Concentration 80% (v/v) has a greater inhibition zone response than other concentrations. The inhibition zone response of *S. mutans* bacteria is greater than that of *L. acidophilus* bacteria due to differences in the roles and characteristics of the two bacteria in the formation of dental plaque and the development of dental caries. *L. acidophilus* and *S. mutans* bacteria are gram-positive

bacteria that produce acid during the demineralization process of tooth structure which causes caries.

L. acidophilus is a probiotic that has the ability to grow in an acidic environment and metabolize sugar from food quickly into organic acids consisting of lactic acid, acetic acid, propionic acid, and butyric acid. Therefore, *L. acidophilus* bacteria are stronger or more resistant to the antibacterial properties of eco-enzymes. *S. mutans* bacteria have the ability to convert sugar in food into acid that damages tooth enamel, forms plaque and triggers demineralization which causes the formation of caries on teeth. Meanwhile, *L. acidophilus* bacteria are bacteria that play a role in the development and continuation of the caries process because these bacteria are more isolated in deep caries, compared to before the development of caries and the beginning of tooth decay. Lactobacillus acidophilus in vegetable juices enhances probiotic content, antioxidant activity, and organoleptic properties, providing lactose-intolerant vegan consumers with healthy non-dairy options. Based on Liu [30] reveals that *Lactobacillus acidophilus* has a wide range of applications in functional and edible probiotic preparations because of its resistance to acid and bile salts and its health-promoting properties in a variety of food products. *L. acidophilus* is more often found in deep caries lesions (advanced caries), so it acts as a secondary bacterium that accelerates caries progression, not as an initial initiator. In contrast, *S. mutans* is very dominant in the early stages of caries formation and the number of colonies is directly proportional to the severity of caries [31]. This explains why the inhibition zone response of *S. mutans* to antibacterial such as eco-enzyme is greater than that of *L. acidophilus* because *S. mutans* is more sensitive, while *L. acidophilus* is more resistant and plays a role in the advanced stage of caries This shows that *S. mutans* bacteria have a major role in the formation of dental caries so that the inhibition zone response of these bacteria was greater than other bacteria [32,33].

MIC and MKC

The diameter of the inhibition zone formed from antibacterial bioactive compounds can only provide information regarding the strength of the inhibition zone of the bioactive compound, but does not provide information regarding the MIC and MKC of compounds that have antibacterial activity which can inhibit or kill the growth of bacteria that cause dental caries. Determination of MIC was observed by looking at turbidity and calculating the difference in optical density ΔOD values in the two test bacteria. The results of the MIC test based on turbidity are shown in **Figure 5**.

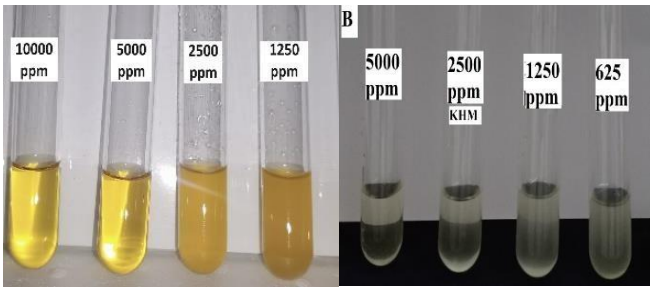

Figure 5. MIC against *L. acidophilus* (A), *S. mutans* (B) based on turbidity

If the turbidity of the test compound tube that has been added with bacteria is the same as the turbidity of the tube in the negative control, then the bacteria can still grow. Therefore, the sample is unable to inhibit the growth of the test bacteria, but if the turbidity of the test compound tube that has been added with bacteria decreases or approaches the turbidity of the tube in the positive control, then the growth of the bacteria has been inhibited. The ΔOD value (optical density difference value) in bacteria that cause dental caries can be seen in **Table 3**, it shows that the minimum inhibitory concentration of eco-enzymes is in the following order: *L. acidophilus* bacteria at concentrations and *S. mutans* bacteria. The concentration of eco-enzyme on *S. mutans* bacteria is higher than that of *L. acidophilus*, which means that *L. acidophilus* bacteria are easier to kill than *S. mutans* bacteria. The determination of the minimum killing concentration is determined from the presence or absence of bacteria growing from the results of the minimum inhibitory concentration determination test [34].

The determination of the minimum killing concentration is carried out to determine the antibacterial properties of eco-enzymes including bacteriostatic or bactericidal which is carried out at test tube concentrations that do not show turbidity in the MIC test in the form of concentrations up to 2500 ppm against *L. acidophilus* bacteria MIC in the form of concentrations of 2500 ppm against *S. mutans* bacteria. The results of the antibacterial activity test by determining the minimum killing concentration are shown in **Figure 6**.

Based on the figure, in MRS agar there were no growth of *L. acidophilus* and *S. mutans*. These are caused by eco-enzyme in the concentrations still have the ability to inhibit bacterial growth and also provide a killing effect or bactericidal. The antibacterial activity of eco-enzyme is related to its acetic acid and

lactic acid content. Organic acids can inhibit and kill the growth of microorganisms through a mechanism, where dissociated and ionized molecules flow through the cell membrane of microorganisms. [34] The results of the study showed that the eco-enzyme mixture of papaya peel waste, orange peel and pineapple peel can inhibit and kill the growth of bacteria that cause dental caries. This shows that the eco-enzyme used in this study is antibacterial, bacteriostatic and bactericidal [35].

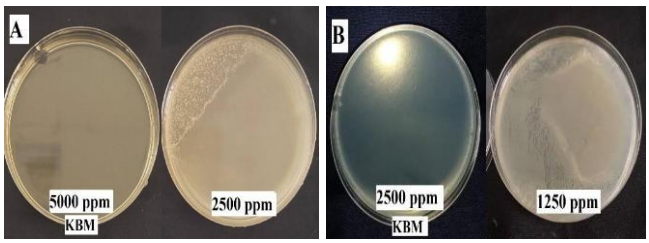

Figure 6. MKC against *L. acidophilus* (A)*, S. mutan* (B)

CONCLUSION

Based on the research, it could be concluded that the characterized form on eco-enzyme derived from papaya peel, orange peel, and pineapple peel exhibited a sour orange peel aroma, a cloudy brown appearance, and a yield of 8.4 liters from 6 liters of water. Chemical analysis revealed acetic acid, lactic acid (total acid: 2.26%), protein (40.45 mg/mL), and secondary metabolites (alkaloids, flavonoids, saponins), alongside enzymatic activities of protease (0.0246 U/mL), amylase (0.0032 U/mL), and lipase (5 U/mL). The eco-enzyme showed strong antibacterial activity against *Streptococcus mutans* (inhibition zone: 23.33 mm) and moderate activity against *Lactobacillus acidophilus* (inhibition zone: 8.23 mm) at 40% (v/v), with MIC and MKC values of 2500 ppm and 5000 ppm, respectively. These findings highlight its potential as a natural antibacterial agent for oral health and sustainable waste management, warranting further research for optimization and practical applications.

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