The influence of cocoa fruit husk extract on the degradation of eps (extracellular polymeric substance) in Lactobacillus acidophilus biofilm

Azimah Regita*

ABSTRACT

Introduction: Dental caries is characterized by progressive demineralization and plaque formation by Lactobacillus acidophilus bacteria due to biofilm formation as a microbial defense mechanism that binds to EPS. To address this issue, cocoa fruit husks extract is utilized as it contains active flavonoids with antibacterial properties. Demonstrate that cocoa fruit husk extract can degrade Lactobacillus acidophilus bacterial EPS biofilm and determine the optimal concentration of cocoa fruit husk extract.

Methods & Materials: Cocoa Fruit Husk Extract Preparation was followed by Lactobacillus acidophilus bacterial preparation. Dilution was performed to achieve a concentration of 106 Bacteria/mL. Microtiter plates with flat-bottom 24 wells were sealed. They were then divided into two groups: one as a control without cocoa fruit husk extract and the other with cocoa fruit husk extract at concentrations of 1.56%; 3.125%; 4.69%; 6.25%; and 7.8%, and incubated at 35°C for 24 hours. Subsequently, they were aspirated, washed 4 times with phosphate-buffered saline, stained with dextran alexa fluor 647, and rinsed with distilled water. Biofilm sections, 0.5 mm thick, were placed on glass slides, and EPS Biofilm was measured using CLSM at 40x magnification.

Results: The division into microtiter plates showed that the 7.8% concentration had the highest potential with a value of $X \pm SD = 8.3366 \pm 1.76364$ in degrading EPS biofilm compared to others. This is in line with previous research, indicating that higher extract concentrations lead to better biofilm degradation activity.

Conclusion: Cocoa fruit husk extract can degrade Lactobacillus acidophilus bacterial EPS biofilm, with the most optimal concentration being 7.8%.

Keywords: Cocoa fruit husks extract, EPS biofilm degradation, Lactobacillus acidophilus
INTRODUCTION

Dental and oral health have developed into a serious matter. This arises from the public’s limited awareness about the importance of maintaining dental and oral well-being, resulting in a spike in dental and oral health issues in the Indonesian community, reaching 90% of the population (Asmawati, et al., 2023).

Tooth decay or Dental Caries, commonly known as cavities, is typically characterized by a gradual demineralization of teeth followed by bacterial metabolic reactions (Listrianah, et al., 2018). The main trigger for the initiation of the tooth decay process is the presence of certain bacteria capable of lowering the oral cavity’s pH to a critical level of 5.5, coupled with inadequate oral hygiene practices (Suratri, et al., 2017).

Tooth decay entails the deterioration of teeth starting from the surface and progressing inward (Maharani, et al., 2022). When the decay gets close to the pulp, it leads to changes such as secondary dentin formation and pulpitis (potentially accompanied by pain) (Kartinawanti & Asy’ari, 2021). It can also result in invasive bacterial action or even pulp death. This infected dead pulp tissue subsequently brings about alterations in the periapical tissue (Fitri, et al., 2023).

Among the 200 types of bacteria isolated from dental plaque, Streptococcus mutans bacteria with serotypes C, E, and F, along with Lactobacillus acidophilus, Bifidobacterium dentium, Actinomyces viscosus, and Streptococcus sobrinus with serotypes C and G, are the most pathogenic (Kaligis, et al., 2017). These bacteria are acid-resistant, capable of surviving in highly acidic environments, and cling to tooth surfaces to metabolize carbs and produce organic acids, causing a drastic drop in oral pH and resulting in enamel demineralization (Radang, et al., 2021). Leftover food containing carbs in the mouth is fermented by the normal oral flora, leading to the production of pyruvic acid and lactic acid through glycolysis. Bacteria like Lactobacillus acidophilus and Streptococcus mutans play a role in this glycolysis process (Elkhaira, et al., 2019).

Lactobacillus acidophilus contributes to the formation of dental plaque, a key factor behind tooth decay. It frequently acts as a culprit for secondary caries lesions, accelerating the demineralization of tooth surfaces (Khadaf, et al., 2021). The low pH is what initiates the process of tooth decay, causing decalcification and tooth deterioration. This contributes to the formation of cavities, as these bacteria form a protective biofilm, defending themselves against antibiotics and immune responses (Subekti, et al., 2019).

Biofilm is an aggregate of microbes, similar or diverse, adhering to biological or non-biological surfaces, where cells attach to one another and the substrate via the extracellular polymeric substance (EPS) matrix. The formation of biofilm follows stages: initial attachment, permanent attachment, maturation I, maturation II, and dispersion stage. Initially, microbes attach to solid surfaces using delicate hair-like structures. During permanent attachment, microbes adhere more firmly, aided by extracellular polymers. In maturation I, other bacteria are pulled in, forming extracellular polysaccharides as bacterial cells continue to grow and develop. In maturation II, accumulated bacteria create multiple layers. Finally, some bacteria disperse and colonize other areas. Bacteria within biofilm exhibit higher resistance to antimicrobials compared to free-floating planktonic forms (Robbani & Wahjono, 2018).

Biofilm maintains its structure by binding together through chains of molecules referred to as EPS or extracellular polysaccharides, forming a polymer that strengthens bacterial adhesion (Muhammad, et al., 2020). Polysaccharides serve not only to adhere to surfaces but also to concentrate food particles present in the surrounding air (Kristianto, et al., 2020).
In the battle against these highly resistant microorganisms, including bacterial spores, the practice of "Toilet of Cavity" is essential. This involves utilizing herbal substances as a substitute for synthetic materials in sterilization (Leksanawati, et al., 2020). The preference for herbal ingredients arises from the reduction in health risks associated with synthetic materials, affordability, and the increased potential for harnessing the benefits of herbal plants such as cocoa (Yassir & Asnah, 2018). Indonesia is the world’s third-largest cocoa producer, steadily growing at 3.5 percent yearly. Nowadays, cocoa plants are predominantly known as the foundational ingredient for crafting chocolate, often linked to being a cavity-causing treat. However, cocoa has a wealth of health benefits due to its cocoa fruits being rich in polyphenolic compounds like catechins, anthocyanins, and proanthocyanidins (Managanta, et al., 2019).

Additionally, the general public tends to perceive cocoa solely as a source of beneficial fruits, mainly overlooking the value of cocoa fruit husks, often considering them waste or animal feed. In truth, cocoa fruit husks constitute the mesocarp or outer part of the cocoa fruit, encompassing the husk and the flesh beneath it before the cluster of fruits (Miranda, et al., 2020). These husks contain active compounds like flavonoids or condensed or polymerized tannins, such as anthocyanidins, catechins, and leucoanthocyanidin, which are tightly linked to glucose (Manalu, 2018). These bioactive compounds have demonstrated antibacterial properties, holding the potential for elevated economic value if utilized effectively. Moreover, cocoa fruit husks are known to contain active alkaloid compounds like theobromine, serving as a calming agent and animal feed (Lestari & Asri, 2021).

Therefore, the aim of this research is to prove that cocoa fruit husk extract can degrade the EPS of *Lactobacillus acidophilus* bacterial biofilm and obtain optimal concentrations of cocoa fruit husk extract.

**METHODS & MATERIALS**

The research was carried out at the Microbiology Laboratory of the Faculty of Medicine and the Central Laboratory of Life Sciences, Brawijaya University with the sample used being the bacterial biofilm stock *Lactobacillus Acidophilus*. In proving that cocoa fruit husk extract can degrade the EPS of *Lactobacillus acidophilus* bacterial biofilm and to obtain optimal concentrations, the research design and procedures are as follows.

1. Research Design / Concept

   ![Research Design](image)

   **Figure 1.** Research design
2. Research Procedures
   a. Making Cocoa Fruit Husk Extract

   The cocoa fruit husk was subjected to a series of processing steps to yield a concentrated extract. Initially, the fruit husk was thoroughly cleaned, sliced thinly, and subsequently oven-dried at a temperature of 50°C. Following this, the dried husk was finely pulverized using a blender, resulting in cocoa fruit husk powder. This powder was then immersed in 96% ethanol at a ratio of 1:7 and allowed to soak for a duration of 24 hours. Subsequently, the solution was subjected to filtration, while the remaining residue was subjected to a second round of soaking for 24 hours, using 96% ethanol at a ratio of 1:4. The solution was again filtered, and the resulting filtrate was combined with the filtrate from the previous step. This combined mixture was subjected to evaporation in a water bath maintained at 50°C, until evaporation of ethanol was achieved, resulting in the obtainment of a concentrated extract.

   b. Preparation of Lactobacillus acidophilus bacterium

   The bacterium Lactobacillus acidophilus was obtained in a quantity of 1 colony-forming unit (CFU) and was subsequently cultured in MRSB (de Man, Rogosa, and Sharpe broth) medium. The culture was incubated at a temperature of 35°C for a period of 24 hours. Following incubation, the bacterial suspension was evaluated for turbidity and adjusted to meet the 0.5 McFarland standard, equivalent to a concentration of 1.5 x 10^8 CFU/ml. This standardization process was achieved by visually comparing the bacterial suspension to a standard 0.5 McFarland turbidity standard, using direct observation against a white background with black lines. If the turbidity of the bacterial suspension did not match the standard, the suspension could be diluted or supplemented with additional bacteria, followed by homogenization.

   c. Making of Lactobacillus acidophilus biofilm

   The bacterium Lactobacillus acidophilus was diluted in Trypticase Soy Broth up to 1:40 ratio and left to incubate overnight, resulting in a bacterial concentration of 10^6 bacteria/mL. Subsequently, 0.1 mL of this culture was taken as the positive control, and 0.2 mL was taken as the negative control. These volumes were dispensed into flat-bottom 24-well microtiter plates and sealed. Next, three microtiter plates were incubated under anaerobic conditions at a temperature of 35°C for a duration of 72 hours. Each microtiter plate contained Lactobacillus acidophilus bacteria and 1% glucose. The number of wells used was adjusted according to the concentrations of the test substances employed, and each well was labeled with the respective concentration of the test substance.

   d. Giving cocoa fruit husk extract to Lactobacillus acidophilus biofilm

   Following the biofilm formation process, the cultures were divided into individual microtiter plates, including a control without cocoa fruit husk extract and varying concentrations of cocoa fruit husk extract at 1.56%, 3.125%, 4.69%, 6.25%, and 7.8%. Subsequently, these microtiter plates were incubated at a temperature of 35°C for a duration of 24 hours. Afterward, the contents of each microtiter plate were aspirated, and they were subjected to four washes using 0.2 mL of phosphate-buffered saline (pH 7.3) to remove planktonic bacteria.

   e. Biofilm Lactobacillus acidophilus colouring

   The microorganisms forming biofilms attached to the microtiter plate were stained using 2 mL of dextran Alexa Fluor 647 in a dark room for a duration of 30 minutes.
Subsequently, they were rinsed with distilled water (aquadest) to remove excess dye.

g. Measurement of EPS biofilm
The biofilm samples were cross-sectionally cut to a thickness of 0.5 mm and placed on prepared glass slides. Subsequently, the measurement of the Biofilm’s Extracellular Polymeric Substances (EPS) was conducted using an inverted Leica TCS-SPE Confocal Laser Scanning Microscope (CLSM) at a magnification of 40x with a pixel format of 1024x1024.

RESULT
In this study, testing was conducted on a microtiter plate, which included a control group without cocoa fruit husk extract and various concentrations of cocoa fruit husk extract (1.56%, 3.125%, 4.69%, 6.25%, and 7.8%), with the aim of degrading the bacterial biofilm of *Lactobacillus Acidophilus*. The results of this testing are presented in Table 1.

<table>
<thead>
<tr>
<th>Table 1. Descriptive Statistics</th>
<th>Sample (N)</th>
<th>Mean (X)</th>
<th>Std. Deviation (SD)</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive_Control</td>
<td>5</td>
<td>306.2708</td>
<td>11.46871</td>
<td>298.43</td>
<td>325.66</td>
</tr>
<tr>
<td>Concentration_1.56</td>
<td>5</td>
<td>64.8774</td>
<td>17.04461</td>
<td>45.04</td>
<td>85.81</td>
</tr>
<tr>
<td>Concentration_3.125</td>
<td>5</td>
<td>18.0808</td>
<td>8.18919</td>
<td>11.45</td>
<td>32.06</td>
</tr>
<tr>
<td>Concentration_4.690</td>
<td>5</td>
<td>115.2522</td>
<td>20.27386</td>
<td>92.19</td>
<td>145.49</td>
</tr>
<tr>
<td>Concentration_6.25</td>
<td>5</td>
<td>15.8616</td>
<td>1.38282</td>
<td>13.84</td>
<td>17.48</td>
</tr>
<tr>
<td>Concentration_7.8</td>
<td>5</td>
<td>8.3366</td>
<td>1.76364</td>
<td>6.09</td>
<td>10.98</td>
</tr>
</tbody>
</table>

From Table 1, it is evident that cocoa fruit husk extract can effectively degrade the extracellular polymeric substances (EPS) of the *Lactobacillus acidophilus* bacterial biofilm. The optimal concentration for this degradation was found to be 7.8%, as it exhibited the highest potential with a mean value of $X \pm SD = 8.3366 \pm 1.76364$ for EPS degradation compared to other concentrations. Additional measurements of EPS in the *Lactobacillus acidophilus* biofilm were conducted using confocal laser scanning microscopy (CLSM) at a 40x magnification, as depicted in Figure 2. The figure includes three image analyses: A. 3D slice, B. Thickness slice, and C. Surface area slice.

![Figure 2. Image analyses of (CLSM) in concentration 7.8](image)

DISCUSSION
This research aims to discover biofilm degradation activity against *Lactobacillus acidophilus* bacteria. The ability to break down biofilms is tied to the compound’s capacity to penetrate the formed biofilm, effectively navigating through the layer of Extracellular Polymeric Substance (EPS) or the mucous layer that envelops the bacteria. Additionally, a critical trait of biofilm degradation compounds is their capability to remove EPS from
established biofilms. Many antimicrobial agents struggle to infiltrate biofilms due to the EPS acting as a barrier that safeguards the bacterial cells within. As a result, compounds that can degrade biofilms by eliminating EPS from already-formed biofilms have emerged as an alternative solution (Ardani, et al., 2010).

This research centers on the bacterial biofilm *Lactobacillus acidophilus*, as these bacteria play a pivotal role in plaque formation, a primary factor contributing to dental cavities (Badet & Thebaud, 2008). *Lactobacillus acidophilus* frequently acts as a catalyst for secondary caries lesions, which can accelerate the demineralization of tooth surfaces (Quivey, 2006). This bacteria produces lactic acid as the end product of carbohydrate fermentation. In the oral environment, it contributes to glucose metabolism by producing organic acids that lower the pH to below 5. This drop in pH triggers decalcification and initiates tooth decay (Sharma, et al., 2008). Such conditions can lead to the formation of dental caries, with the bacteria forming biofilms as a defense mechanism against antibiotics and immune responses (Noguchi, et al., 2005).

In this study, we utilized cocoa fruit husks extract to test its effects on the bacterial biofilm of *Lactobacillus acidophilus*. The research was conducted through in vitro experimental methods in a laboratory setting, aiming to determine the impact of cocoa fruit husks extract on the degradation of *Lactobacillus acidophilus* bacterial biofilm at specific concentrations. The study involved the use of cocoa fruit husks extract at five different concentrations: 1.56%, 3.125%, 4.69%, 6.25%, and 7.81%. These concentrations were obtained through a serial dilution method to establish the effective concentrations of cocoa fruit husks extract for degrading *Lactobacillus acidophilus* biofilm. The initial serial dilution process started from concentrations of 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.56%, and 0.78%. Then, the two most promising concentrations from the preliminary research, 3.125%, and 6.25%, were selected. Three additional concentrations were chosen to continue the investigation: one below 3.125%, one above 6.25%, and one between 3.125% and 6.25%, using the same intervals. By performing calculations using the same interval approach, the resulting concentrations were 1.56%, 3.125%, 4.69%, 6.25%, and 7.8%.

Biofilm researchers often encounter challenges when conducting microscopic analyses due to interference from fluorescent signals of the used stains and the dense nature of the biofilm layers. Consequently, the Confocal Laser Scanning Microscope (CLSM) was chosen for reading *Lactobacillus acidophilus* biofilms in this study due to its high accuracy in assessing bacterial quantity, components, surface area, and biofilm thickness (Parlina, 2019). Staining using Alexa Fluor 647 dextran conjugate was also employed to visualize biofilm EPS. The distribution resulting from the red-colored polysaccharide indicates the high polysaccharide concentration within the biofilm. Alexa Fluor 647 dextran was chosen for this research due to its ability to bind with biofilm EPS, emitting red fluorescence under excitation and emission wavelengths of 460 nm and 650 nm, respectively (Homenta, 2016).

The research results reveal that at concentrations of 1.56%, 3.125%, 4.69%, 6.25%, and 7.8%, the amount of EPS in the biofilm is lower compared to the control group. This indicates that cocoa fruit husks extract has degradation activity on *Lactobacillus acidophilus* biofilm. This finding is further supported by phytochemical tests, which yielded results showing that cocoa fruit husks extract contains substances like flavonoids, alkaloids, and tannins. Each concentration was tested with five repetitions. Significant differences in EPS quantity were observed compared to the control group, aligning with the theory that a substance with antibiofilm properties can break down biofilms through various mechanisms, including penetrating the extracellular matrix, dispersing cells from the biofilm, or destabilizing the EPS in the biofilm (Donlan, 2022).
The 3D slice image illustrates the intensity values of EPS within the biofilm when observed through a 3D slice. Blue color indicates the remaining EPS, while yellow also signifies remaining EPS, with dextran conjugate staining still adhering to the EPS. The intensity values increase as the image becomes more yellow, and the numerical values can be seen on the left side of the image. The Thickness slice image displays the intensity values of EPS within the biofilm when observed through a thickness slice. Thinner biofilm thickness indicates more effective degradation by cocoa fruit husk extract, and intensity values can be identified from the numerical values on the left side of the image. Finally, the Surface area slice image presents intensity values of EPS when observed through a surface area slice. This image reveals red-colored spots, indicating remaining EPS that has been stained with dextran conjugate. A reduced presence of these red spots implies greater effectiveness of cocoa fruit husk extract in degrading EPS within the biofilm.

Furthermore, the graph displaying the Mean and Standard Deviation (SD) of EPS in the *Lactobacillus acidophilus* biofilm can be seen in Figure 3, with data sourced from Table 1. As previously elucidated, the optimal concentration for this study was 7.8%, which exhibited the highest potential for degrading EPS biofilm when compared to concentrations of 1.56%, 3.125%, 4.68%, and 6.25%. This finding aligns with Loresta (2012), which suggests that higher extract concentrations lead to improved biofilm degradation activity.

![Figure 3. Mean and std. deviation of EPS biofilm Lactobacillus acidophilus](image)

**CONCLUSION**

In summary, the experimental results obtained from the microtiter plate testing, including a control group without cocoa fruit husk extract and various extract concentrations, demonstrate that cocoa fruit husk extract has the capacity to degrade the EPS biofilm of *Lactobacillus acidophilus* bacteria. The most optimal concentration for this degradation was determined to be 7.8%, as it exhibited the highest potential among all concentrations, with a mean value of $X \pm SD = 8.3366 \pm 1.76364$ for EPS degradation.
REFERENCES


