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Ecoenzymes as Biocatalysts: Accelerating Biofilm Formation in Wastewater Biofilters

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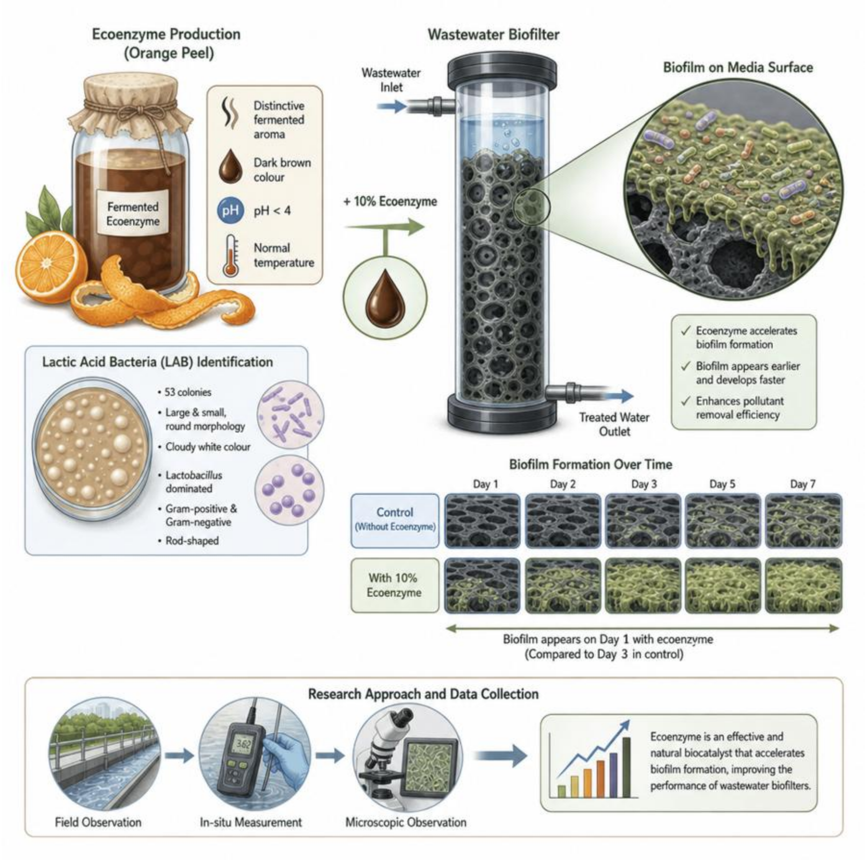
ABSTRACT

Biofilters have advantages in waste treatment, but limitations related to the formation time of biofilms, which play an important role in reducing pollutants in wastewater. The addition of biocatalysts such as ecoenzymes can be an innovative solution in accelerating biofilm formation. This study aimed to examine the effectiveness of adding ecoenzyme to biofilter in accelerating biofilm formation. This research is quantitative with an experimental approach using a completely randomized design (CRD), The test was conducted by adding 10% orange peel ecoenzyme, where the characteristic test shows that this ecoenzyme is better quality than those made from vegetable waste. Data collection was carried out through: (1) Field observation, (2) In-situ measurement, and (3) Microscopic observation. The data was analyzed descriptively to examine the quality of ecoenzymes, meanwhile, biofilm development was observed microscopically. The result found the ecoenzyme has a distinctive fermented aroma, is dark brown colour, pH < 4 and normal temperature, this indicates that the ecoenzyme is of good quality. Identification of lactic acid bacteria (LAB) revealed 53 colonies with large and small round morphology and a cloudy white colour. Lactobacillus bacteria dominated, both Gram-positive and Gram-negative found, rod-shaped, and large round colonies. Based on microscopic observation, biofilm formation in the control group was slower than in the samples treated with 10% ecoenzyme, a biofilm formation began to appear on the first day. While in the control samples it only appeared on the third day. This indicates that the ecoenzyme addition may accelerate biofilm formation.

Key Messages:

- The application of orange peel-derived ecoenzymes as a biocatalyst significantly accelerates early biofilm formation in wastewater biofilters—reducing the initial onset time from three days to a single day—thereby offering an innovative, sustainable approach to optimizing wastewater treatment efficiency.

GRAPHICAL ABSTRACT



INTRODUCTION

Household activities ranging from cooking, washing, bathing and other needs will produce wastewater, where 80% of clean water used will end up as wastewater (1). Population growth will have an impact on the increase in the quantity of domestic liquid waste each year. The amount of wastewater produced has not been accompanied by individual or communal treatment efforts, which has resulted in a decline in surface water quality, soil pollution and an increased risk of waterborne diseases. Domestic wastewater is rich in organic material, which has the potential to cause blooming and dead zones in water bodies. Waste that is directly discharged into the environment without treatment will cause a decline in environmental quality (2).

The government's capacity to provide communal wastewater treatment facilities remains limited, so domestic wastewater management needs to be improved. As a first step, the public needs to be introduced to appropriate technologies with simple designs, low operating costs, and ease applied in wastewater management. Wastewater can be treated physically, chemically, and biologically, but biological treatment is a modern alternative treatment method that plays a major role in improving human health and maintaining water quality (3). Based on the characteristics of nutrient-rich domestic wastewater, the biofilter method can be used, which utilizes natural microorganisms in wastewater in the process of degrading pollutants.

The biofilter (biofilm filter) method has limitations, such as the time required for biofilm formation on the filter media, which is quite long (4). Under normal conditions, biofilm formation and maturation require 3-4 weeks. The length of time it takes to form a biofilm will slow down the waste treatment process, while wastewater is always produced. Wastewater treatment with a biofilter system is carried out by flowing wastewater into a reactor filled with media where biofilm adheres to the surface of the media. The biofilm method is currently being considered in wastewater treatment for pollutant degradation and reducing carbon production (5,6).

Biofilms generally begin to form within 1-2 weeks after operation but achieving mature and

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optimally functioning biofilms requires a longer time, even up to several months. The time required for biofilm formation varies depending on several factors, namely the type of microorganism, environmental conditions, especially pH and water temperature, and the type of biofilter media (7). Simple technology for household wastewater management is urgently needed, prompting researchers to develop a biofilter method incorporating ecoenzymes as catalysts for domestic wastewater treatment.

Currently, studies related to the application of orange peel ecoenzymes in biofilters are still limited, so further research is needed both at the laboratory scale and in pilot-scale applications. Ecoenzymes contain various enzymes that have the potential to accelerate the biofilm formation (8). The substrate content in ecoenzymes as a source of nutrients for bacteria can stimulate bacterial growth and colonization. Ecoenzymes also have a low pH (acidic), which can create an environment conducive to the growth of lactic acid bacteria, which plays an important role in biofilm formation (9).

Biofilm is a complex community of microorganisms that attach to media or surfaces and are embedded in an extracellular matrix consisting of polysaccharides and proteins. This complex matrix forms on the surface of the filter media. The presence of a solid support surface is beneficial for biofilm formation and serves as support for the attachment of microorganisms (10). This biofilm can protect microorganisms from environmental stress and unfavorable conditions such as drought, temperature changes, lack of nutrients, exposure to toxic contaminants, and better survival compared to planktonic microorganisms (11).

The effect of ecoenzymes on biofilm formation can vary, depending on the composition of the ecoenzyme raw materials and the organic load in the wastewater. Different substrate compositions will produce different characteristics, bioactive compounds, and enzymes. These differences in substrate content in ecoenzymes and organic load in wastewater result in varying effects on biofilm growth. The thickness of Anabaena flos-aquae microalgae biofilm, nutrient removal efficiency, and EPS production increased with increasing nutrient load in synthetic wastewater in laboratory tests (12). Samples with high organic loads, microalgal biofilms with a thickness of 75 μm showed the best performance in C, N, and P removal efficiency, possibly due to high nutrient loads promoting high biological activity. Other studies found that nutrient regulation optimizes cell synthesis and EPS secretion through changes in element accumulation, particularly C/N and Ca, which increased by 84% and 112%, respectively. Biofilm loss due to external environmental stress was reduced by 50-75%, indicating increased biofilm stability (13).

These studies conclude that adequate nutrition stimulates microbial growth, increasing both cell numbers and EPS production, resulting in thicker and more robust biofilms with greater pollutant reduction capacity. Accelerated biofilm formation and maturation means that biofilters can also reach their optimal treatment capacity more quickly. Based on this description, the researcher was interested in examining the effectiveness of adding ecoenzyme catalysts to accelerate biofilm formation, which also means increasing the efficiency of biofilters.

METHODS

This is a quantitative research with an experimental design using a completely randomised design approach. (14) defines experimental research intended to determine whether there are any effects of treatment on the subjects under investigation. The filter media used is bioball, which is used because it has a large surface area and its round shape reduces the potential for clogging. Ecoenzyme is made from organic waste with the following composition: (a) a mixture of vegetable scraps and (b) orange peels. Wastewater samples were collected from residential areas around complex X using grab sampling methods. Samples were taken between 6:00 and 7:00 a.m., considering that peak household activities such as cooking, bathing, and washing generally take place at that time.

Organoleptic testing of ecoenzyme quality for color and aroma parameters, temperature and pH were measured in situ with a portable Ez-9909 pH meter. BAL identification included macroscopic characteristic testing based on (15), followed by microscopic examination involving gram staining, with reference to (16). Microscopic observation of biofilm formation was conducted at the Laboratory of the Central Sulawesi Provincial Environment Agency. Biofilm formation was conducted in an anaerobic biofilter reactor (10 L capacity) using bioballs as a media. Observations were made on control samples

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(without ecoenzyme) and treatment samples (with 10% orange peel ecoenzyme added). Observations of the biofilm formed on the bioballs using electron microscope were conducted on days 1–30. The research data will be analyzed descriptively and presented in the form of tables, images/graphs, and narrative. pH and temperature measurements are tabulated in a table and plotted in a graph where the X-axis represents monitoring time, and the Y-axis represents pH and temperature values.

RESULTS

Identification of Characteristic Ecoenzymes

Organoleptic test and in situ test

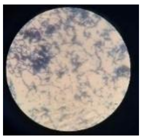
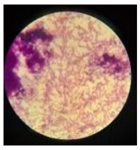
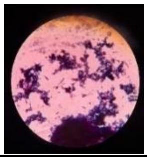
Color and aroma characteristics were observed organoleptically, while pH and temperature were measured using a portable pH meter. The test results are presented in table 1. The observations showed that both ecoenzymes were of good quality.

Table 1. Characteristics of Ecoenzymes

Sample Ecoenzyme	Color	Aroma	pH	Temperature (°C)
Orange peels ecoenzyme	Yellowish brown	Distinctive aroma of fermentation	3.24	27.9
Vegetable mixture ecoenzyme	Dark brown	Distinctive aroma of fermentation	3.86	26.6

Based on macroscopic and microscopic tests on orange peel ecoenzyme, Lactobacillus sp. was revealed with 53 colonies in the form of large and small round shapes with a pale white color. In addition, a gram-negative bacterium, Streptobasillus sp., was also found. Meanwhile, in vegetable ecoenzyme, Lactobacillus sp. was also found, as well as the pathogenic bacteria Eschericia coli and Streptobasillus sp. The data from the ecoenzyme observation can be seen in table 2.

Table 2. Bacteria Identification in Orange Peels and Vegetable Mixture Ecoenzyme

Sample code	Species	Cell Shape	Colony Shape	Coloni Colour	Gram	Picture
Orange Peels Ecoenzyme						
Sample 1	*Lactobacillus*	Stick-shaped	Large sphere	Murky white	+	
Sample 2	*Lactobacillus*	Stick-shaped	Large sphere	Murky white	-	
Sample 3	*Streptobacillus*	Stick-shaped	Small sphere	Murky white	-	
Sample 4	*Lactobacillus*	Stick-shaped	Large sphere	Murky white	+	

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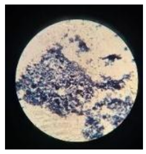
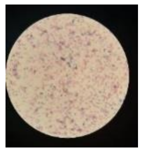
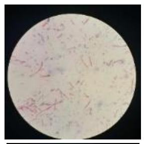
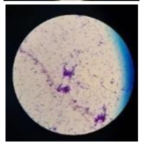
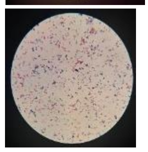
Sample code	Species	Cell Shape	Colony Shape	Coloni Colour	Gram	Picture
Sample 5	*Staphylococcus aureus*	Round (coccus)	Small round	Cloudy white	+	
Vegetable Mixture Ecoenzyme						
Sample 1	*Eschericia coli*	Stick-shaped	Large sphere	Murky white	-	
Sample 2	*Lactobacillus sp*	Stick-shaped	Large sphere	Murky white	-	
Sample 3	*Eschericia coli*	Round (coccus)	Small round	Cloudy white	+	
Sample 4	*Streptobacillus sp*	Stick-shaped	Large sphere	Murky white	-	
Sample 5	*Eschericia coli*	Round (coccus)	Small round	Cloudy white	-	

Biofilm Formation

The formation of biofilm was observed microscopically, and biofilm formation tests were conducted simply by immersing bioballs (filter media) in control samples consisting of domestic wastewater and treatment samples consisting of a mixture of domestic wastewater and 10% ecoenzyme. The ecoenzyme used in this experiment was orange peel ecoenzyme, considering its lower pH value and has more colonies of lactic acid bacteria. In the experiment, biofilm formation was observed with pH and temperature as control parameters, as these two parameters greatly affect bacterial activity. Based on field identification, the control sample group that was not treated with ecoenzymes showed very limited changes.

The water remains turbid with a predominantly dark gray color, indicating low biological activity capable of breaking down organic particles. A pungent odor is detected from the outset and does not decrease significantly, indicating that the decomposition process is slow. The water temperature remained stable between 28.2°C and 29.6°C, but this was not sufficient to stimulate active microbial dynamics. The pH of the water fluctuated only slightly between 8.05 and 8.20, with no spikes indicating microorganism growth. In the control sample, a biofilm layer formed slowly on the third day, and the water became clearer after the sixteenth day. In contrast, in the treatment with the addition of 10% Ecoenzyme, visual changes in the water began to appear on the seventh day, and a biofilm layer on the bioball media had appeared since the first day of treatment.

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A B

Figure 1. Comparison of biofilm formation after 2 weeks (Figure A without ecoenzyme & Figure B with ecoenzyme)

DISCUSSION

Based on organoleptic testing, both orange peel ecoenzyme and vegetable mixture have different characteristics. Although they have the same aroma, their color, pH, and temperature are different. Orange peel ecoenzyme has a more acidic pH, which makes it better for use in the next stage of research. Temperature and pH both of ecoenzyme in table 1 still complies with the recommendations for good ecoenzyme quality (17). This ecoenzyme has a distinctive fresh sour aroma characteristic of fermentation. The sour aroma produced comes from the acetic acid contained in the ecoenzyme liquid (18). Acetic acid generally gives a sour taste and aroma to liquids or food. Acetic acid is produced from the metabolic process of bacteria that naturally exists in fruit and vegetable waste.

The anaerobic metabolic process, commonly known as fermentation, is an attempt by bacteria to obtain energy from carbohydrates in anaerobic conditions (without oxygen) and with by-products in the form of alcohol or acetic acid (depending on the type of microorganism) (19). Fungi and some types of bacteria produce alcohol in the fermentation process, while most bacteria produce acetic acid. This fermentation process is the result of the activity of enzymes contained in bacteria or fungi. The color of ecoenzyme was a brownish yellow and dark chocolate, the fermentation of ecoenzyme can be said to be successful if a brownish solution is formed. This is in accordance with the results of the experiment, where the colour of the ecoenzyme product changed from a clear brown (the original colour of the brown sugar solution changed to a brownish colour. The brownish colour is due to the original colour of the brown sugar used, which is brown, then mixed with the pulp/residue from papaya and cucumber peels (20).

Microscopic observation showed that lactic acid bacteria were found in both samples, but more colonies were found in the orange peel ecoenzyme sample. Of the five samples examined, three contained Lactobacillus sp, so this sample can be used for biofilm formation testing on bioball media. The presence of lactic acid bacteria inhibits the growth of pathogenic microorganisms by producing lactic acid, thereby lowering the pH of the liquid (9). The fermentation process also produces certain enzymes depending on the available substrate. Previous studies have also found lipase, cellulase and amylase enzymes in ecoenzyme samples made from orange peel, banana, pineapple and pomegranate (21). These enzymes have the potential to be purified and utilised in other applications. These degradation products serve as energy substrates for microorganisms that form biofilms, thereby accelerating the growth of microbial colonies on the surface of the bio ball media. This finding is in line with research by (8, 22), which states that the natural enzymes in Ecoenzyme can significantly reduce COD and BOD levels by accelerating the activity of decomposing microbes. Enzyme biocatalytic activity is influenced by the optimal pH in the decomposition process of organic materials. However, the performance and stability of this enzyme are greatly influenced by environmental factors such as pH and temperature (23,24). During the research process, observations of temperature showed stability in the range of 27–29 °C, which supports enzyme activity well. In ecoenzyme solutions, the presence of enzymes helps break down proteins, carbohydrates, and fats, which are the main components of organic waste (25,26).

Microscopic observation shows that the biofilm formed is thicker on media soaked in wastewater with 10% ecoenzyme than on media without the addition. This indicates an increase in the microorganism population and accumulation of Extracellular Polymeric Substances (EPS) that act as natural adhesives between microbial cells. Abundant EPS strengthens the biofilm structure and enhances pollutant binding efficiency on the media surface. The substrate in ecoenzymes as a source of bacterial nutrients can stimulate bacterial growth and colonisation, increasing both cell count and Extracellular Polymeric

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Substance (EPS) production, thereby producing a thicker and more robust biofilm with greater pollutant reduction capacity. This biofilm forms when bacteria or other suitable microbes find the right conditions, attach to a solid surface, multiply, and form colonies in the form of EPS (27,28).

The formation and maturation of a biofilm normally takes 3 - 4 weeks (4). It generally begins to form within 1-2 weeks after operation, but it takes more than 30 days to reach maturity and function properly (29). In this study, it was found that in samples treated with 10% ecoenzyme, biofilm formation began to appear on the first day. Meanwhile, in the control samples, biofilm formation only appeared on the third day. This shows that the addition of ecoenzyme may accelerate biofilm formation. The rate of biofilm maturation is a crucial factor because biofilm is the main component in biofilter systems that are responsible for degrading organic pollutants (30). This acceleration indicates that ecoenzymes act as effective bio-activators in supporting the initiation and growth of microbial communities on the surface of filter media.

The mechanism of accelerated biofilm formation by ecoenzymes can be explained through several aspects, such as coenzymes, which are the result of organic waste fermentation, rich in hydrolytic enzymes such as protease, lipase, and amylase, as well as organic acids (31,32). These enzymes function as biocatalysts that accelerate the decomposition of complex macromolecules in wastewater, such as proteins, fats, oils, and carbohydrates, into simpler compounds that are easily assimilated by microorganisms (32, 33). Thus, the availability of more easily degradable substrates facilitates microbial metabolism and promotes faster bacterial population growth on the filter medium, which ultimately accelerates biofilm formation and maturation (34). The pH (8.05–8.20) and temperature (28.2–29.6°C) in the biofilter reactor during the biofilm formation monitoring process can support the production and activity of lipase, amylase, and protease enzymes from mesophilic microorganisms, particularly bacterial strains such as Bacillus spp. that produce alkaliphilic enzymes. Temperatures within this range fall within the mesophilic range typical for microbial growth and enzyme secretion, so these thermal conditions allow for enzyme synthesis without significant heat stress. A pH of around 8.0 is favorable for alkaline enzymes such as alkaline proteases and many bacterial lipases, which exhibit optimal activity at neutral to alkaline pH (35).

Additionally, the acetic acid and propionic acid content in ecoenzymes plays a role in creating a microenvironment conducive to the growth of beneficial bacteria while inhibiting pathogenic microorganisms (36, 37). The acidic pH of ecoenzymes can also affect the initial conditions in biofilters, potentially selecting microbial populations that will form biofilms, supporting the dominance of acid-tolerant bacteria or those whose enzyme activity is optimal at a certain pH range (38). The stable and nutrient-rich environment resulting from partial degradation by ecoenzymes allows microorganisms to attach and replicate more efficiently, forming a strong and thick biofilm layer in a shorter time. This acceleration of biofilm maturation has significant practical implications. A shorter biofilm initiation time means that biofilters can reach their optimal treatment capacity faster, reducing operational waiting time and increasing pollutant reduction efficiency early on (39, 40, 41). Thus, the addition of 10% ecoenzyme has proven to be a promising strategy for improving the overall performance of anaerobic biofilters.

Biofilm formation occurs in four stages, namely (a) Adhesion or initial attachment, (b) Colonisation is the initial development of the biofilm structure, with microcolony formation occurring immediately after bacteria attach to the substrate surface. (c) Maturation, (d) Biofilm spread occurs when bacteria leave the biofilm regularly (42). Biofilm maturation occurs when cells have strongly adhered to the substrate surface. During the maturation stage, the biofilm will continue to grow in line with colony growth. The biofilm will continue to develop with an increase in size and change in shape. At this stage, the biofilm thickness is >100 µm and can reach 300–400 µm (43). the optimal biofilm thickness for nitrification is around 100–200 µm, while for nitrification-denitrification the thickness must be at least 500 µm (44). The faster the biofilm matures and can work optimally, the more it is expected to increase the efficiency of the biofilter in wastewater treatment, particularly in decomposing organic material found in wastewater. In this study, observation of biofilm thickness was not conducted. Further research using the CLSM method is needed to obtain more detailed information regarding the effect of ecoenzyme addition on the duration of biofilm formation and maturation, which is assessed based on biofilm thickness. However, this

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microscopic observation can serve as an initial indicator of biofilm maturation. The observation shows that adding ecoenzyme can accelerate biofilm formation, which in turn automatically accelerates the maturation process.

CONCLUSION

Observations on both samples of ecoenzyme found lactic acid bacteria namely Lactobacillus sp. Both ecoenzymes also meet the characteristics of a quality ecoenzyme, but the orange peel ecoenzyme has a more acidic pH value so it can be used as a biocatalyst to accelerate biofilm formation. The addition of 10% ecoenzyme was proven to accelerate biofilm formation. In the treated sample, biofilm began to form on the first day, while in the control group, biofilm only formed on the third day. This study concludes that the addition of 10% ecoenzyme has the potential to be developed as a biocatalyst to accelerate biofilm formation by reducing the initial biofilm formation time from 3 days to 1 day.

DECLARATION OF THE USE OF AI

The authors declare that no artificial intelligence (AI), AI-assisted technologies, or large language models (LLMs) were used in the conception of the study, data analysis, or the drafting, writing, and editing of this manuscript. The only exception is the graphical abstract, which was created using the design platform Illustrae (https://illustrae.co/). The authors take full responsibility for the content and accuracy of the graphical abstract and the entire manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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