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The Effect of Variations in Sugar Types and Fermentation Time on Enzyme Activity and Total Titrated Acid on Eco-Enzyme Results of Fermentation

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ABSTRACT
Eco-enzyme (EE) production is an alternative for reducing organic waste such as fruit peels and vegetables. EE is widely used as natural organic fertilizer, purification of contaminated groundwater, pesticides, herbicides, and natural insecticides. EE is made through a fermentation process for 3 months with a certain ratio of the composition of organic matter, sugar, and water. This study aimed to determine the effect of the type of sugar and the fermentation time on the raw material for the fermentation of the enzyme activity and total acid titrated. The fermentation product showed that the lipase activity in the use of white sugar and brown coconut sugar decreased with increasing fermentation time. While the molasses continues to increase with the optimum enzyme activity in the third month 20.33 U/mL. The percentage of total acid titrated in white sugar and molasses is higher than in brown coconut sugar, where the total acid in molasses continues to increase.

Keywords: *Eco-Enzyme, Fermentation, Lipase Activity, Total Titrated Acid*

1. INTRODUCTION

Indonesia is an agricultural country with abundant vegetable and fruit products. The amount of organic products also contributes to the increase in the amount of organic waste. Data from the Ministry of Environment and Forestry 2020 states that food wastes account for 36.69% of the total Indonesian national waste. As much as 31.65% of the waste comes from household waste. Optimal and integrative waste management is needed so that organic waste can be utilized and does not pollute the environment.

One alternative to treat fruit and vegetable waste is by making eco-enzymes (EE) which was initiated by Rosukon Poompanvong [1]. Eco-enzyme (EE) is made through a fermentation process for 3 months with a ratio of organic matter, sugar, and water composition of 3: 1: 10. EE is widely used as natural organic fertilizer, purification of contaminated groundwater, pesticides, herbicides, and natural insecticides [2]. The skin of the fruit contains various nutrients, namely macronutrients in the form of minerals and micronutrients such as carbohydrates, proteins, lipids, and fiber. The mineral content in the fruit skin is zinc, manganese, iron, calcium, potassium, sodium, phosphate, and magnesium [3].

The EE product contains a consortium of microbes, organic acid compounds, enzymes, and various secondary metabolites. Types of secondary metabolites in EE include flavonoids, steroids, quinones, alkaloids, and organic acid saponins [4]. In the fermentation process, microbes also secrete various types of enzymes such as catalase, amylase, protease, pectinase, glucose, isomerase, cellulase, hemicellulase, lipase, and lactase [5]. The conversion of substrates and nutrients into

various compounds in the metabolic process is part of the microbial defense mechanism from the environment.

Sugar as one of the main raw materials for fermentation plays an important role in providing carbon sources in the form of sucrose, fructose, and glucose. In Supriyani's research [6], variations in sugar produce different yields. Different types of sugar also affect the microbes that produce extracellular enzymes such as lipase, amylase, and cellulase. Molasses is widely used by the community because it is considered a waste of cane sugar production. Meanwhile, coconut brown sugar and white sugar are types of sugar that are easily available and are often used by the public. White sugar, coconut brown sugar, and molasses have different sucrose content of 99.3; 61; and 40% [7].

Fermentation conditions such as fermentation time are the main determinants of fermentation results. Microbes will produce various types of organic acid enzymes and secondary metabolites for their metabolic processes. At a certain time, the acidic conditions in the fermentation also increase along with the high levels of acetic and lactic acids produced and accumulated. The acidic conditions of fermentation have the probability to affect the activity of enzymes secreted by microbes. Enzymes are proteins that can be denatured due to several factors, one of which is acidic conditions. Therefore, this study aimed to determine the effect of the type of sugar and the length of fermentation on the raw material for fermentation on enzyme activity and total titrated acid.

2. METHODOLOGY

2.1. Material and Reagents

The ingredients used to make the Eco Enzyme extract in this research were a mixture of orange, melon, watermelon, guava, apple, and dragon fruit peels. Sugars used in this study were table sugar, brown sugar, and molasses. Other materials and reagents used were olive oil, phosphate buffer, sodium hydroxide, acetone, ethanol, phenolphthalein, and oxalic acid.

2.2. Eco-Enzyme Production

First, fruit peels were washed and cut into small pieces. Then, three types of Eco Enzyme were prepared in different containers and harvested at the age of 1, 2, and 3 months of fermentation. Eco Enzyme filtrates were filtered and stored for future analysis.

2.3. Lipase Activity Determination

One gram of olive oil was added into Erlenmeyer then followed with 4 ml of phosphate buffer pH 6.5 and 1 ml of the sample. The mixture was homogenized for 10 minutes in a water bath at 45^{o}C then added a solution of ethanol: acetone (1:1) and 3 drops of phenolphthalein indicator. The mixture was titrated with 0.1 N NaOH until the pink color become colorless. The total volume used of NaOH was recorded for calculating the enzyme activity. The enzyme activity was calculated by the Equation (1), where EA is enzyme activity, Vs is volume of sample titration, Vb is volume titration blank, Ve is volume of enzyme used, and n is Normality of NaOH.

$$EA = \frac{(Vs \times Vb) \times n \times 1000}{Ve \times t} \quad (1)$$

2.4. Total Titrated Acid Determination

The total acid was determined by taking 10 mL of diluted sample in an Erlenmeyer flask and adding 2 drops of phenolphthalein indicator. Then, the mixture was titrated with standardized 0.1 NaOH. The total acid in percentage was determined by the formula (2), where TTA is the total titrated acid, a is volume of NaOH titration, b is normality of NaOH, c is dilution factor, and d is sample weight.

$$TTA = \frac{a \times b \times c}{d} \times 100\% \quad (2)$$

2.5. Data Analysis

The sample was analyzed in triplicated and expressed as mean with standard deviation (SD). Data obtained were analyzed by one-way ANOVA and the Tukey– Honestly Significant Differences (Tukey's HSD) test. All tests were set at a significant level of 0.05 [8].

3. RESULT AND DISCUSSION

3.1. Lipase Activity

The level of microbial survival in the fermentation process depends on environmental conditions including the availability of nutrients in the media. Microbial metabolism in the growth process in the logarithmic phase is assisted by enzymes. In the fermentation process, enzymes are produced along with the production of other primary metabolites such as amino acids, organic acids, and vitamins. The types of enzymes produced by several microbial strains are amylase, protease, cellulase, pectinase, and lipase [9].

Lipase enzyme (triacylglycerol acyl hydrolase) is a hydrolase enzyme that catalyzes triglycerides into glycerol and free fatty acids. Lipase also plays a role in the transesterification process of esters so that it is widely used in various fields such as food processing, detergents, cosmetics, organic synthesis, and the pharmaceutical industry [10]. The use of different types of sugar and harvest time showed different enzyme activities. The lipase enzyme was tested by utilizing the specific action of lipase in hydrolyzing lipids into free fatty acids which were then titrated using an alkaline solution such as KOH or NaOH and phenolphthalein as an indicator. The amount of free fatty acids indicates the amount of lipase activity in the sample [11].

Table 1. Lipase Activity of Eco-Enzyme at Different Fermentation Time and Sugar Type

Sugar Type	Lipase Activity (U/mL)*		
	1 Month	2 Month	3 Month
White Sugar	37.33±0.58^{c}	17.33±1.15^{ab}	12.67 ± 0.58^{b}
Brown Coconut Sugar	13.33±0.58 ^{a}	14.00 ± 1.00^{a}	6.33 ± 0.58^{a}
Molasses	18.33 ± 0.58^{b}	18.00 ± 0.00^{b}	20.33 ± 0.58^{c}

*Data are means of 3 replications. Values followed by the different letters, within the sugar variation in every month, were significantly different in Tukey's honestly significant differences (HSD) test with ($p<0.05$).

The results of the analysis of lipase activity shown in Table 1 with the harvest period of 1, 2, and 3 months of fermentation using white sugar resulted in the activity of 37.33; 17,33 and 12.67 U/mL. Meanwhile, brown sugar produced activities of 13.33; 14.00, and 6.33 U/mL. In molasses, the lipase enzyme activity was 18.33; 18.00, and 20.33 U/mL. White sugar produces the greatest initial activity of the other three types of sugar. However, lipase activity tends to decrease in the 2nd and 3rd months.

3.2. Total Titrated Acid

Fermentation of the manufacture of eco-enzymes using fruit peels can produce various products of organic acid compounds. Some of these organic acids are citric, lactic, malic, and oxalic acids [1]. The acid can come from the organic material used in the fruit skin. The total acid value in papaya peel in carboxymethyl cellulose hydrocolloid is 10.25% [12], while pineapple peel is 22.78 g/L in fermentation with 10% sucrose content [13]. In addition, organic acids also come from the secondary metabolism of fermenting agent microorganisms, such as *Lactobacillus plantarum*, *Aspergillus niger,* and the *Acetobacter* group of bacteria. The increase in the amount of acid during the fermentation process can affect the enzyme activity and the metabolism of the microorganism itself.

Table 2. Total Titrated Acid of Eco-Enzyme at Different Fermentation Time and Sugar Type

Sugar Type	Total Titrated Acid (%)*		
	1 Month	2 Month	3 Month
White Sugar	14.67±0.58^{b}	13.00 ± 0.00^{b}	13.67 ± 0.58^{b}
Brown Coconut Sugar	6.00 ± 1.00^{a}	5.67 ± 1.15^{a}	4.67 ± 0.58^{a}
Molasses	14.33±1.15^{b}	13.67 ± 0.58^{b}	17.00 ± 0.00^{b}

*Data are means of 3 replications and followed by the different letter, within the sugar variation in every month, were significantly different in Tukey's Honestly Significant Differences (HSD) test with ($p<0.05$).

The amount of organic acid that can be produced can be determined by using the total acid titration method. Samples that have been dripped with phenolphthalein indicator will change color to purplish after being titrated with NaOH [14]. This color change is caused by the resonance of the electron isomer. Each acid-base indicator is an ion that has a different ionization constant.

The results of the analysis of the percentage of the total acid product of eco-enzyme after harvest in the 1, 2, and 3 months are shown in Table 2. The percentage of total acid tends to be almost the same in the types of white sugar and molasses. Both produce 14% acid in the first and second months. In the third month, there was an increase in the amount of acid. Meanwhile, brown coconut sugar showed a lower yield which only produced 6% total acid.

In Arun's research [1], the 15th day of EE fermentation produced acetic, lactic, oxalic, malic, and citric acids with concentrations of 11.12; 26.02; 44.81; 11.05, and 39.05 g/L. After the 90th day, the concentration of acetic acid increased to 78.14 g/L, while the concentration of other acids decreased. While in Rasit's research [15], the levels of acetic acid produced from EE of tomato and citrus waste reached 14.13 g/L and 35 g/L with acidity levels at pH 2.79 and 2.86.

3.3. Sugar Effect in Eco-Enzyme Production

Some microbes like glucose and fructose so that other sugars cannot be processed when there are enough glucose and fructose available. This allows the rate of production of ethanol and acid compounds to increase [16]. Acid production on fermentation is related to acetic and lactate acid synthesis in microbial metabolism.

Several types of microbial lipases decreased activity at a glucose concentration of 10 mM. There are *Aspergillus niger*, *Mucor javanicus*, *Rhizopus oryzae*, *Candida lipolytica*, *Penicillium roqueforti*, and *Geotrichum candidum*. The decrease in activity starts at 5 mM glucose and stagnates at 10 mM to 20 mM [17].

In molasses sugar, the value of lipase activity tends to increase. This is also accompanied by an increase in total acid. However, in brown coconut sugar, lipase activity decreased, followed by a decrease in total acid but not significant. Meanwhile, white sugar has a trend where the lipase activity decreases while the total acid tends to be stable. Based on Tukey's HSD analysis, it was shown that in the first and third months, all variety of sugar types had a significantly different value on the results of lipase enzyme activity. In the second month, only brown coconut sugar and molasses had significantly different enzyme activity.

3.4. Fermentation Time Effect in Eco Enzyme Production

The tendency of decreasing enzyme activity was in contrast to the increasing percentage of total titrated acid in the eco-enzyme product. In the 1st, 2nd, and 3rd month fermentation period, the use of white sugar produced a total acidity of 14.67; 13 and 13.67%. The use of brown sugar produces a total acid of 6; 5.67 and 4.67%. While the molasses produces the amount of acid by 14.33; 13.67 and 17%. Tukey's HSD statistical analysis of total titrated acid showed that in the first and second months, brown coconut sugar was significantly different from other sugars. While white sugar was not significantly different from molasses. However, in the third month, the all variety of sugar types were significantly different.

In lipase production, incubation time has an important role. The incubation period was 4 days in submerged fermentation by *Rhizopus sp*. ZAC3 can produce lipase optimally [18]. The optimal incubation time was almost the same for *Fusarium solani, Rhizopus arrhizus, Penicillium notatum, Genoderma lucidium,* and *Emericella nidulan*. Several other organisms such as *Rhizopus chinensis* and *Aspergillus niger* MTCC 2594 require 3 days to produce lipase optimally. The mechanism of action of the lipase enzyme consists of acetylation and deacetylation processes involving the amino acids serine, histidine, and aspartate.

A decrease in enzyme activity followed by an increase in total acid content indicates protein denaturation due to the influence of acid. Denaturation causes changes in the configuration of protein molecules without causing damage to peptide bonds. Under acidic conditions, proteins can precipitate and their solubility in water increases when treated with excess acid. In Triyono's research [19], acetic acid and citric acid can precipitate 54-73% - 61.95% protein with an optimum pH of 4.5. The enzyme has structural and functional stability at pH 4 -10 [20].

The type of sugar used in the manufacture of eco-enzymes through the fermentation process affects the enzyme activity in the product. Molasses sugar produces the greatest enzyme activity than other types of sugar. However, the lipase enzyme activity decreased in the use of white and brown coconut sugar with increasing fermentation age. Meanwhile, the percentage of total titrated acid is increasing in uses of molasses but tends to be stable in white and brown coconut sugar.

AUTHORS' CONTRIBUTIONS

Imam Abu Hanifah conducted research with ideas and suggestions from Arie Srihadyastuie and Sasangka Prasetyawan. The research plan was tested and given additional suggestions by Tri Ardyati and Anna Safitri.

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