

# Antioxidant activity of coenzyme derived from Siamese orange peel (*Citrus nobilis*) at different fermentation duration

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## ABSTRACT

**Background:** Ecoenzyme is an organic fermentation product derived from fruit peel waste, offering zero-waste system benefits along with antibacterial, insecticidal, and cleaning properties. Kintamani, a major producer of Siamese oranges (*Citrus nobilis*) in Bali, generates significant orange peel waste suitable for ecoenzyme production. However, the antioxidant potential of ecoenzymes remains poorly studied.

**Objective:** This study aimed to evaluate the antioxidant activity of ecoenzymes produced from Kintamani Siamese orange peel waste, enhancing their beneficial value.

**Methods:** This experimental study assessed and compared the antioxidant activity of ecoenzymes fermented for 10 days, 1 month, and 3 months. Antioxidant activity was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, with results expressed as IC<sub>50</sub> values.

**Results:** Antioxidant activity, classified according to Blois criteria, showed the highest activity after three months of fermentation. The mean IC<sub>50</sub> values were 53,612 ppm for 10-day fermentation; 18,753 ppm for 1-month fermentation; and 6,727 ppm for 3-month fermentation.

**Conclusion:** Ecoenzymes derived from Kintamani Siamese orange peel waste exhibited very weak antioxidant activity across all fermentation durations. A 3-month fermentation period is recommended to achieve the highest antioxidant activity compared to shorter durations.

**Keywords:** antioxidant activity, ecoenzyme, orange peel waste, Siamese orange

## Introduction

According to the National Waste Management Information System (SIPSN), national waste production in 2021 reached 23,636,290 tonnes per year, with organic waste accounting for 27.5% of the total. The largest source of organic waste is domestic household waste, which includes food scraps, fruit peels, and vegetable stems [1]. Among these, fruit waste presents significant potential for reuse. Reprocessing domestic household waste is essential to mitigate waste production and its associated environmental and health risks.

In 2003, Dr. Rosukon Poompanvong, a Thai scientist, developed an organic fermentation solution from fruit peel waste, now widely known as ecoenzyme. Fruit peels, including citrus peels, can serve as raw materials for ecoenzyme production [2]. However, the specific use of citrus peels, such as orange peels, in ecoenzyme production remains underexplored. Citrus peels are rich in organic compounds that can influence the properties of ecoenzyme products. Research has shown that ecoenzymes produced from orange peels after four weeks of fermentation achieve a pH of 3.8 and

contain high levels of citric acid, which enhances the release of hydrolytic enzymes compared to other materials [3].

Bali Province is a notable citrus-producing region in Indonesia. In particular, Kintamani District is the largest producer of Siamese oranges (*Citrus nobilis*) in Bali, with a reported production of 104,528 tons in 2021 [4]. Utilizing Siamese orange peel waste for coenzyme production not only supports zero-waste initiatives but also adds value to domestic agricultural output [5].

Ecoenzyme is a biochemical product derived from the fermentation of organic waste, with the fermentation duration tailored to specific production goals. While many studies recommend a fermentation period of three months [6–8], research by Kamaruddin et al. (2019) demonstrated that fermentation over just 8–10 days can yield coenzymes with an ideal pH below 4.0 [9]. Ecoenzymes have diverse applications, including as antibacterial agents, insecticides, and cleaning solutions. Their acidic content, comprising alcohol and acetic acid, makes them effective as natural pesticides and household cleaning agents. Furthermore, coenzymes possess antibacterial properties due to antioxidant compounds such as alkaloids, polyphenols, flavonoids, and saponins [10].

However, studies exploring the antioxidant activity of coenzymes are limited, particularly regarding those derived from Siamese orange peels. Previous research found that coenzymes fermented from papaya, pineapple, and citrus lime for 10 days demonstrated stronger antioxidant activity than those fermented for three months [11]. Despite these findings, no research has specifically investigated the antioxidant properties of coenzymes made exclusively from Siamese orange peels.

This study aims to analyze the antioxidant activity of coenzymes produced from Kintamani Siamese orange peel waste using fermentation durations of 10 days, 1 month, and 3 months. The fermentation duration is defined as the time from sealing the container holding the mixture of fruit peels, sugar, and water (ecoenzyme solution) to the point of filtration.

## Method

This experimental study employed a post-test-only design to measure the antioxidant activity of coenzymes derived from Kintamani Siamese orange peel (*Citrus nobilis*) fermented for 10 days, 1 month, and 3 months. Antioxidant activity was assessed using the DPPH method. Ethical clearance was obtained from the Ethics Commission of the Faculty of Medicine and Health Sciences, Warmadewa University (Ethics Number: 381b/Unwar/FKIK/EC-KEPK/XII/2023).

## Sample

The coenzyme samples were prepared using Siamese orange peel, granulated sugar, and water in a ratio of 3:1:10. Oranges were selected based on specific criteria, including a harvesting age of 240–270 days post-bloom, freshness, cleanliness, firmness, and freedom from pests, diseases, foreign aromas, or tastes. A total of 10 kg of oranges was collected, yielding 1650 grams of peels for coenzyme production.

## Ecoenzyme production

Oranges were sourced from Kintamani District, Bangli, and subjected to washing and sorting based on inclusion criteria. The peels were separated, cut into small pieces, and dried using clean cloths. For each fermentation duration (10 days, 1 month, and 3 months), 100 grams of orange peel were combined with 300 grams of sugar and 1 liter of water in airtight plastic containers. The mixtures were stirred thoroughly, sealed, and occasionally opened to release gas buildup. Each container was labeled to indicate the fermentation duration, and three replicates were prepared for each group. The resulting coenzymes were analyzed for antioxidant activity.

## Antioxidant activity testing

Ecoenzyme samples were tested at concentrations of 200 ppm, 400 ppm, 600 ppm, 800 ppm, and 1000 ppm. Each sample (3 ml) was mixed with 1 ml of 2,2-diphenyl-1-picrylhydrazyl

(DPPH) solution (100  $\mu\text{M}$ ) and incubated in the dark at 30°C for 30 minutes. Absorbance was measured using a UV-Vis spectrophotometer at 517 nm [12, 13]. Antioxidant activity was expressed as percentage inhibition, calculated using the formula:

$$\% \text{ Inhibition} = \frac{\text{Blank absorbance} - \text{Sample absorbance}}{\text{Blank absorbance}} \times 100\% \dots (1)$$

Blank absorbance was determined using a solution of 3 ml methanol and 1 ml DPPH solution (100  $\mu\text{M}$ ). Linear regression analysis ( $y = a + bx$ ) was performed, where  $x$  represents the concentration (ppm), and  $y$  represents percentage inhibition. The  $\text{IC}_{50}$  value, the concentration required to inhibit 50% of DPPH activity, was derived by substituting  $y=50$  into the regression equation. Antioxidant activity was classified as follows [14]: very strong:  $\text{IC}_{50} < 50$  ppm, strong:  $50 \text{ ppm} \leq \text{IC}_{50} < 100$  ppm, moderate:  $100 \text{ ppm} \leq \text{IC}_{50} < 150$  ppm, weak:  $150 \text{ ppm} \leq \text{IC}_{50} < 200$  ppm, and very weak:  $\text{IC}_{50} > 200$  ppm. A lower  $\text{IC}_{50}$  value indicates higher antioxidant activity.

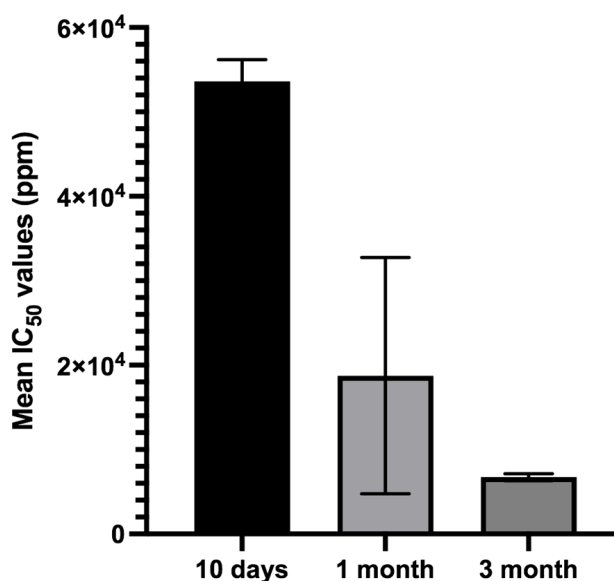
### Data analysis

$\text{IC}_{50}$  values were expressed as mean  $\pm$  standard deviation and visualized using bar charts for comparison. Results were interpreted according to Blois's classification.

### Results

Antioxidant activity testing was conducted using the DPPH method, which measures the reduction of DPPH radicals upon interaction with ecoenzyme solutions derived from Siamese orange peels fermented for varying durations. Despite testing, no significant visible color changes were observed in the DPPH solution after adding the ecoenzyme samples. Each fermentation group was tested in triplicate, with ecoenzyme concentrations of 200 ppm, 400 ppm, 600 ppm, 800 ppm, and 1000 ppm. The absorbance of the control (blank) solution was measured at 0.469.

Percentage inhibition values were calculated for each concentration, and a linear regression equation ( $y = ax + b$ ) was used to determine  $\text{IC}_{50}$



**Figure 1.** Mean of  $\text{IC}_{50}$  values of values of ecoenzyme with different fermentation duration

values, where  $x$  represents concentration (ppm) and  $y$  represents the percentage inhibition (%). The  $\text{IC}_{50}$  value, indicating the concentration required to achieve 50% inhibition, was calculated by substituting  $y=50$  into the regression equation.

The  $\text{IC}_{50}$  values for ecoenzyme solutions at fermentation durations of 10 days, 1 month, and 3 months. Figure 1 presents the mean  $\text{IC}_{50}$  values as follows: 10-day fermentation:  $53,612 \pm 2,596$  ppm; 1-month fermentation:  $18,753 \pm 14,004$  ppm; 3-month fermentation:  $6,727 \pm 415$  ppm. Based on Blois's classification, ecoenzymes derived from Siamese orange peels exhibit very weak antioxidant activity across all fermentation durations. Among the groups, the lowest antioxidant activity was observed for the 10-day fermentation period, while the highest antioxidant activity was recorded for the 3-month fermentation period, as indicated by the lower  $\text{IC}_{50}$  value.

### Discussion

Ecoenzyme represents a practical application of the zero-waste system, which seeks to significantly reduce or eliminate waste by repurposing organic materials [15]. Produced from fruit and vegetable residues combined with sugar and water in a ratio of 3:1:10, ecoenzyme serves multiple purposes,

including as a natural cleaning agent, disinfectant, insecticide, and drain cleaner [16]. These benefits arise from the presence of alcohol and acetic acid, which possess antibacterial properties and can inhibit pathogen growth [17].

The fermentation process is integral to coenzyme production. Fermentation is an anaerobic metabolic process where bacteria metabolize carbohydrates to produce by-products such as alcohol and acetic acid, depending on the microorganism [7]. In this study, fermentation durations of 10 days, 1 month, and 3 months were tested. Ideal coenzyme characteristics, such as a fresh acidic smell, pH below 4, and dark brown color, develop during fermentation [18, 19]. During the early stages of fermentation, alcohol is produced, while acetic acid becomes dominant in later stages, resulting in a distinct acidic odor [19]. Prior research has demonstrated consistent pH levels and gradual changes in color and organic compound concentrations as fermentation progresses [20].

Antioxidant activity testing using the DPPH assay showed weak antioxidant activity in coenzymes across all fermentation durations. The DPPH method is widely used for its simplicity, sensitivity, and reproducibility [21]. In this assay, DPPH, a stable free radical with a characteristic dark purple color, reacts with antioxidants to reduce its free radical activity, leading to a color change measurable by spectrophotometry. However, in this study, no significant color changes were observed, indicating that the coenzymes lacked strong antioxidant properties.

The IC<sub>50</sub> values, indicating the concentration needed to inhibit 50% of radical activity, were relatively high for all fermentation durations, reflecting weak antioxidant activity. Among the durations, the 3-month fermentation exhibited the highest antioxidant activity (lowest IC<sub>50</sub>), followed by the 1-month and 10-day durations. These results align with findings from Luo et al. (2023), who observed increased concentrations of organic acids, total phenolics, and flavonoids during prolonged fermentation [23]. However, earlier studies reported stronger antioxidant activity in

shorter fermentation durations (e.g., 10 days), particularly for coenzymes made from papaya, pineapple, and citrus lime [11].

The variation in antioxidant activity across different fruits and fermentation durations may stem from differences in the chemical composition of raw materials, the rate of antioxidant release, and microbial activity. Citrus peels, including Siamese orange peels, are rich in flavonoids and other antioxidants like hesperidin and naringin, which require longer fermentation to release or form active compounds [24, 25]. In contrast, fruits like papaya and pineapple release antioxidants more rapidly due to higher vitamin C content and simpler molecular structures [26]. Microbial activity also influences the breakdown of organic materials, with complex substrates like orange peels requiring more time for enzymatic degradation [27, 28].

During fermentation, hydrolytic enzymes such as amidases and esterases play a crucial role in breaking down complex organic materials into simpler, bioactive components. These enzymes help release polyphenols, flavonoids, and other antioxidants trapped within plant cell structures [29, 30]. Moreover, fermentation can transform bound antioxidant compounds (e.g., flavonoid glycosides) into more potent free forms (aglycones), enhancing their antioxidant potential [32, 33].

This study's limitations, including a small sample size and potential variability in raw material quality, may have influenced the findings. Factors such as ripeness, freshness, or contamination of orange peels could have affected the levels of antioxidants and the efficiency of fermentation.

Further research is recommended to conduct phytochemical testing for a comprehensive analysis of secondary metabolites, which may explain differences in antioxidant activity and reveal additional functional properties. Studies profiling enzyme activity and microbial communities during fermentation could optimize coenzyme production and applications. Comparative studies with other citrus varieties may also help identify alternative raw materials with greater antioxidant or enzymatic potential.

## Conclusion

Ecoenzymes derived from Siamese orange peel exhibit weak antioxidant activity across fermentation durations of 10 days, 1 month, and 3 months. Among these, a 3-month fermentation duration yielded the highest antioxidant activity, although still limited compared to shorter fermentation periods for other fruits. Despite their weak antioxidant properties, these ecoenzymes hold potential as natural cleaning agents, soil enhancers, or odor neutralizers, contributing to sustainable waste management practices.

## Acknowledgment

We would like to express our sincere gratitude to Agriculture Laboratory of Universitas Warmadewa for their invaluable support and assistance throughout this research.

## Funding

Not applicable.

## Author contributions

AARP and PANKP conceptualized the research, designed the study, and drafted the manuscript. AARP and PANKP also oversaw the preparation of the ecoenzyme samples. SP carried out the antioxidant activity measurements, while AL performed the data analysis. All authors made substantial contributions to the research and collaborated in preparing the final manuscript.

## Declaration of interest

None declared.

Received: July 3, 2024

Revised: September 12, 2024

Accepted: September 18, 2024

Published: December 27, 2024

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