

## Investigation of Changes of Antioxidant Properties of Coffee through Fermentation by Using *Saccharomyces Cerevisiae* and *Bacillus Subtilis*

Nhi Y. Dinh<sup>1</sup>, Duy Q. Nguyen<sup>2</sup>, Phu H. Le<sup>1\*</sup>

<sup>1</sup>Department of Food Technology, School of Biotechnology, International University, Vietnam National University, Ho Chi Minh City, Vietnam

<sup>2</sup>Department of Food Technology, Faculty of Chemical and Food Technology, Ho Chi Minh City, University of Technology and Education, Vietnam

\* Corresponding author. Email: [lhphu@hcmiu.edu.vn](mailto:lhphu@hcmiu.edu.vn)

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### ABSTRACT

The study was carried out to investigate the changes of antioxidant properties and quality of green coffee beans fermented by using *Bacillus subtilis* and *Saccharomyces cerevisiae* as starter cultures in sugarcane and banana juice at different times fermentation. The fermentations were conducted with different juice concentrations for different fermentation times 24, 48, and 72 hours at room temperature. Antioxidant properties were determined by Folin-Ciocalteu reagent for the determination of total phenolic content, DPPH assay for the determination of antioxidant capacity, and colorimetric method used for determination of total flavonoid content. As a result, coffee fermentation at 10°Brix, 10<sup>7</sup> cells/mL for each type of microbe, and the time (48 hours) had a positive effect on antioxidant properties. However, there was not a significant difference in terms of antioxidant properties between fermented and original coffee beans at optimal conditions. Therefore, antioxidant properties were dramatically reduced during the early stages of fermentation, but it was shown to be able to overcome it through this study. This study is a premise to applying microbial products through fermentation to create a coffee with high antioxidant activity compared to conventional coffee products.

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### 1. Introduction

Coffee is described as the world's most popular beverage. Coffee is mainly grown in more than 70 countries by farmers, especially in tropical developing countries, including Vietnam, where the countries are mostly concentrated. However, coffee consumption is distributed across the globe, with a significant percentage kept by developed countries because of its stimulant effect and exquisite taste [1], [2].

Antioxidants are substances that aid in the control of oxidative molecules thanks to their ability to deactivate or suppress the formation of free radicals. Antioxidant therapy reduces oxidative stress which is an imbalance between free radicals and antioxidant defenses by stabilizing or deactivating free radicals before causing any damage to cells. Antioxidants are generally present in nature. Most components of plants, including fruits, seeds, nuts, barks, roots, herbs, and leaves, are particularly susceptible [3]. Furthermore, most antioxidants are absorbed by the body through foods and beverages such as fruits, vegetables, grains, nutritional supplements, tea, juice, coffee, etc. Coffee has been widely recognized as a significant source of antioxidants and other bioactive chemicals, and it is consumed regularly all over the world [4].

Post-harvest coffee processing has a major effect on the quality of coffee. Fermentation of coffee beans is a second processing step that improves the functionality of the beans by increasing antioxidants and sensory qualities [5], [6]. Additional processing methods, including soaking in fruit extracts and fermentation, can improve the functionality of green coffee beans. Lim et. al. was observed that the antioxidant properties increased after soaking green coffee beans in the mulberry extract [7]. *Saccharomyces cerevisiae* is a type of yeast which are responsible for producing alcohol in anaerobic condition and contributing to the winey and fruity aroma in final roasted coffee [8]. *Bacillus subtilis* is

a facultative anaerobic bacterium with the ability to hydrolyze cellulose and hemicellulose which are major obstacles to the enzymatic conversion to free fermentable sugars [9]. It also consumes sugars to produce organic acids (lactic acid, acetic acid, butyric acid, and other carboxylic acids) [10]. These acids are responsible for proteins in coffee beans to break down into amino acids, leading to the decrease in bitterness, astringes of coffee, and contributing to the formation of flavors [11].

In this study, banana and sugarcane juices worked as the nutritional medium and substrate for microbes. Banana and sugarcane diverse nutritional compounds like carbohydrates, protein, minerals, and vitamins, especially soluble sugars, which were the nutritional medium for the growth and transformation of *Saccharomyces cerevisiae* and *Bacillus subtilis*. Therefore, the change of the input medium in this case is an alternative medium that provides nutrients for the fermentation of microorganisms and promotes the fermentation process. This research aimed to innovate a novel type of fermented coffee that has higher antioxidant properties compared to conventional ones. The changes of antioxidant properties were due to the effect of fermentation on the antioxidant compounds of coffee beans [12].

## 2. Materials and Methods.

### 2.1. Materials

The green beans of *Coffea robusta* (*Coffea canephora*) were purchased from Gia Lai provinces, Vietnam. Its moisture content of its was about 11-13%. The quality of coffee beans was uniform in size, and shape, and was not contaminated by insects.

*Bacillus subtilis* was isolated from shell and green coffee beans provided by Phu and the group's members, a product from a study in 2012 [13]. *Saccharomyces cerevisiae* was selected from the microbial collection of the Food Technology Department, International University, Vietnam National University in Ho Chi Minh City.

### 2.2. Methods

#### 2.2.1. Preparation of microorganisms

*Bacillus subtilis* and *Saccharomyces cerevisiae* were inoculated in sterilized MRS agar for 48 hours at 37°C and Czapek Dox agar for 48 hours at 25°C, respectively. These were used as starter cultures in coffee fermentation with juices from banana and sugarcane and were incubated at room temperature under anaerobic conditions [2], [5].

#### 2.2.2. Preparation of nutritional medium

Banana and sugarcane juice were bought from the market and then ground together with a ratio of 1:2 (w/V). And then the mixture was measured Brix degree by refractometer. After that, dilution with distilled water to reach the needed Brix degree.

#### 2.2.3. Investigation of fermentation time

100 grams of green coffee beans were soaked in water for 4 hours with a ratio of 2:1 (V/w) which aims to imbibition the process of dried coffee beans. Coffee was fermented with  $10^7$  cells/mL for each type of microbe and juice at 10°Brix for different durations: 24, 48, and 72 hours. The control sample was unfermented coffee (original green coffee beans) [2], [14].

#### 2.2.4. Investigation of the amount of juice

100 grams of green coffee beans were soaked in 200 mL of water for 4 hours with a ratio of 2:1 (V/w) which aims to imbibition the process of dried coffee beans. The amount of juice was evaluated through the Brix degree. The sample was treated by 3 levels: 5, 10, and 15°Brix (5B, 10B, 15B). The fermentation was inoculated with  $10^7$  cells/mL and the time was obtained from the previous experiment. There are two controls in this factor: one control (C) was the original green coffee beans, and the other was a control (C1) treated with the same procedure as the fermented coffee beans but without juice [2], [15].

#### 2.2.5. Extraction of coffee

After fermentation, the coffee beans were washed with water and dried for 12 hours at 40°C until the moisture content of the coffee beans reached 11%. Then, they were roasted at 240°C for 14 mins. The roasted coffee was ground and sieved to achieve uniform particle size. The ground coffee was separated

into sieve plates with 1mm (No.18). For the extraction, each sample with 10 grams of ground coffee was stirred well with 50 mL boiling water for 15 mins, filtered through the filter paper to harvest coffee extract [16].

#### 2.2.6. Determination of antioxidant activity

The antioxidant activity of the extracts was determined using the technique published by Haile et al., 2020, with some modifications, based on the scavenging activity of the stable 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) [17]. 0.1 mL of the coffee extract with 100 times dilution were mixed with 3.9 mL of 0.075 mM DPPH solution and kept in the dark for 30 mins and then the absorbance was measured at 515 nm by using a UV – visible spectrophotometer. The concentration of DPPH is calculated from the Trolox standard curve and expressed as  $\mu\text{mol}$  Trolox equivalents per g dry weight of the sample ( $\mu\text{mol TE/g DW}$ ).

#### 2.2.7. Determination of total polyphenol contents

Total polyphenol content (TPC) of coffee extracts was determined using Folin - Ciocalteu reagent, as previously described by Haile et al., 2020, with some modification [17]. First, 10mL of Folin-Ciocalteu reagent was diluted with 90 mL distilled water. The coffee extracts were diluted 50 times with distilled water. The reaction was prepared by 2.5 mL diluted Folin- Ciocalteu reagent and 0.2 mL distilled sample extract. The sample was mixed and held for 5 mins. Then, 2 mL of 7.5%  $\text{Na}_2\text{CO}_3$  (w/v) was mixed and incubated in the dark for 1 hour. The absorbance was taken at 765 nm wavelength by using a UV–visible spectrophotometer after 1 hour. The concentration of TPC is calculated from the Gallic acid standard curve and expressed as mg Gallic acid equivalents per g dry weight of the sample (mg GAE/g DW).

#### 2.2.8. Determination of total flavonoid contents

Total flavonoid content (TFC) of each coffee extract was calculated according to the protocol defined by Haile et al., 2020, with some modifications [17]. 0.5 mL of coffee extract was diluted 50 times, 2 mL distilled  $\text{H}_2\text{O}$ , and 0.15 mL of 5%  $\text{NaNO}_2$  were mixed. After 5 mins, 10%  $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$  solution (0.3 mL) was added and incubated for 6 mins. 1 mL of 1N NaOH was added and incubated for 11 mins. Instead of coffee extract, the distilled water was replaced and used as a blank. The absorbance of the sample was determined at 510 nm by a UV/visible spectrophotometer. The total flavonoids in coffee were measured in milligrams of quercetin equivalent per gram of dry weight (mg QE/g DW).

#### 2.2.9. Statistical analysis

All the data were duplicated, collected, and analyzed by calculating, and drawing graphs by Microsoft Office Excel 2016. Analysis of variance (ANOVA), using SPSS statistical methods with Duncan standard, was performed to identify significant differences among samples.

### 3. Results and Discussion

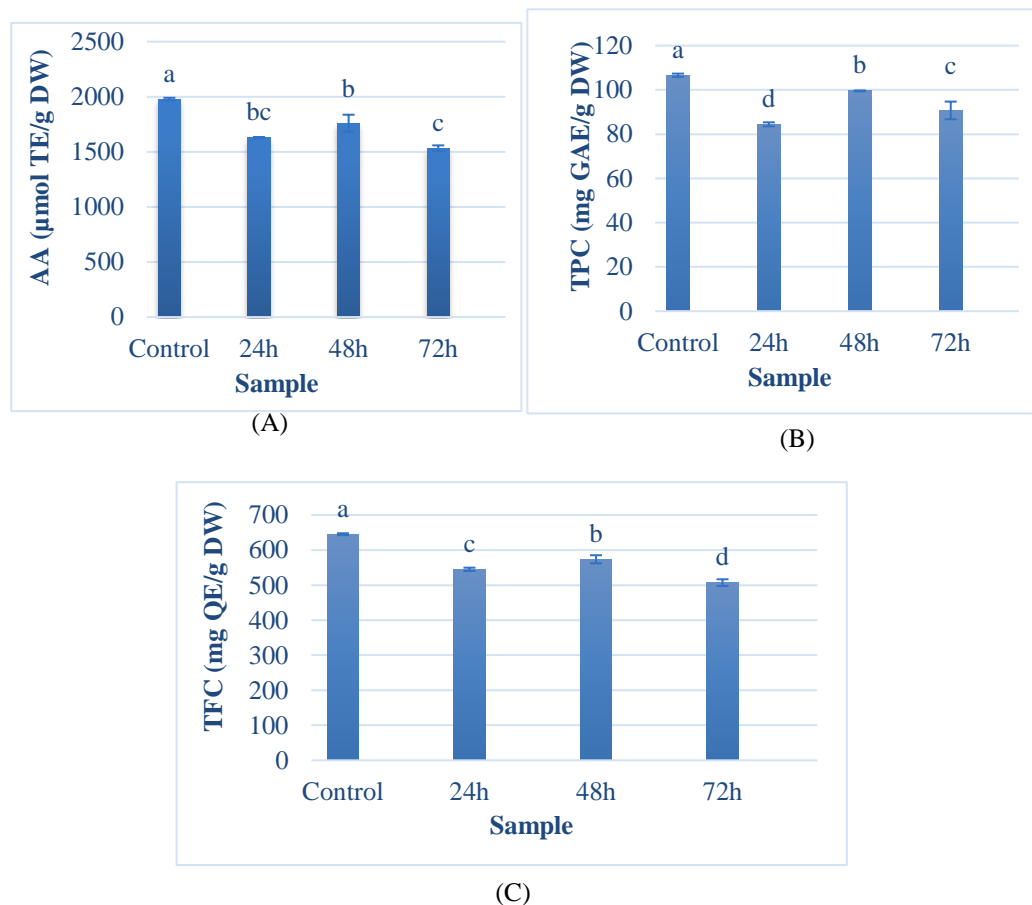
#### 3.1. Effect of fermentation time on antioxidant properties in coffee beans

The experiment was prepared to investigate three periods (24, 48, and 72 hours) for changing antioxidant properties and the quality of coffee. Figure 1 describes the effect of varying fermentation time on antioxidant properties. Based on the result of the experiments, the biotransformation by the *S. cerevisiae* and *B. subtilis* for 48 hours was effective in the significant increase of the antioxidant activity, total polyphenol content, and total flavonoid content when compared to the fermented coffee for 24 and 72 hours. However, when compared to the non-fermented coffee (original green coffee beans or the control) with fermented coffee for 48 hours, a decrease in antioxidant properties was observed.

In the study, the antioxidant potential of the analyzed green coffee extract and fermentation of a combination of *S. cerevisiae* and *B. subtilis* with banana and sugarcane juice was assessed. The examined material has been demonstrated to have a substantial antioxidant capacity, and the fermentation process had a substantial impact on the proportion of free radicals scavenged by the assessed extracts. In all the extracts studied, there was a shift in antioxidant potential over time. The antioxidant potential of the green coffee extract was the highest at 1977.90  $\mu\text{mol TE/g DW}$ . However, green coffee extract treated to a 48-hour fermentation procedure was also found to have substantial antioxidant properties at 1760.05  $\mu\text{mol TE/g DW}$  that is shown in figure 1A. This is similar to green

coffee beans fermented by kombucha [17] and is also observed in the case of tea fermentation [18], [19] and other fermented materials such as ginseng, tea, and soy [20], [21], [22].

As the same trend with antioxidant activity, total polyphenol, and flavonoid content had the highest content at 48-hour fermentation at 99.5 mg GAE/g DW and 573.80 mg QE/g DW, respectively. However, these values were lower than those found in the green coffee extracts (TPC 106.63 mg GAE/g DW and TFC 645.52 mg QE/g DW). This is because esters bound of phenolic compounds which are attached to the cell wall can be broken by fermentation, leading to an increase in their concentration and consequently their functional properties [23, 24] and making them easier to extract after roasting [6]. Depolymerization of the polymerized active compounds might be the reason why longer fermentation time may increase the concentration of polyphenolic compounds. In 24-hour fermentation, TPC decreased might be due to the appearance of polymerized compounds with high molecular weight and limited water solubility. The fermentation time was extended to 48h which could increase TPC concentration because of the depolymerization and the appearance of compounds with higher solubility. When increasing fermentation time to 72 hours, TPC was decreased due to the action of the polyphenol oxidase enzyme which performed the diffusion of phenolics in cell liquids and oxidizes them [5], [17], [25], [26]. The change in TFC might be due to the conversion of insoluble phenolic compounds into soluble flavonoids during fermentation [6], [27] and caused by the increase in acid values during this process, which liberates the bound of flavonoid components and makes them more bioavailable like the reported result about okra seeds in 2014 by Adetuyi, & Ibrahim [26].



**Figure 1.** Effect of fermentation time on antioxidant properties of green coffee beans. Antioxidant activity (A), total polyphenol content (B), and total flavonoid content (C) of fermented coffee extracts.

\*Different letters above the bars indicate a statistically significant difference at  $p < 0.05$  among treatments.

\*\*Control, unfermented coffee; 24h, fermented coffee for 24; 48 hours, fermented coffee for 48; 72 hours, fermented coffee for 72 hours.

However, when compared to the unfermented coffee (original green coffee beans) was compared to fermented coffee for 48 hours, a decrease in antioxidant properties values was observed. The result was not similar to previous studies' trends [6], [17]. After fermentation, the antioxidant properties at the highest result are slightly lower than unfermented green coffee beans. In the first 24 hours, there was a small amount of oxygen in the flash. In aerobic conditions, *S. cerevisiae* consumed oxygen to create H<sub>2</sub>O, and CO<sub>2</sub> as well as increase the number of yeast cells. By contrast, in this condition, *B. subtilis* is inactivated form. Therefore, in the first 24 hours, the fermentation did not occur leading to soluble antioxidant compounds eluted into the medium, making the antioxidant properties decrease after fermentation. At 48 hours and 72 hours, antioxidant properties decreased, maybe because of the over-fermentation. At this time, the microorganisms occurred in the death phase, so the number of living cells decreases and population growth slows dramatically, making the quality of fermentation inadequate. Moreover, during this period of time, *S. cerevisiae* consumed sugar, and the accumulation of large amounts of ethanol produced during fermentation which is toxic for most competing microorganisms as well as organic acids which were produced by *B. subtilis*, inactivate the growth of *S. cerevisiae*.

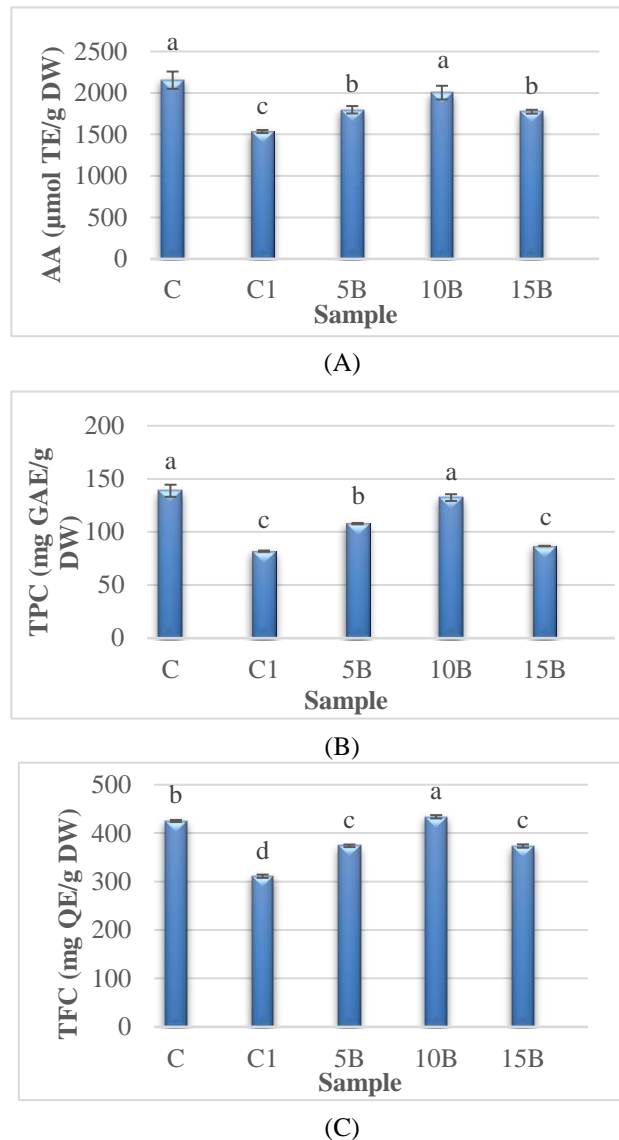
In conclusion, the best fermentation time for this process may be determined from 24 to 48 hours instead of from 24 to 72 hours. So, further study should be considered in the investigation of fermentation times between 24 and 48 hours.

### 3.2. Effect of amount of juice (Brix degree) on antioxidant properties in coffee beans

The experiment was prepared to survey the amount of juice which was based on the sugar content of an aqueous solution via Brix degree for investigation of changes of antioxidant properties through fermentation: 5, 10, 15 °Brix, and two controls. The antioxidant properties of the coffee extract are presented in Figure 2. In general, the antioxidant properties of the fermented coffee extracts significantly changed.

The antioxidant properties of the fermented coffee extracts by using a juice mixture after 48 hours of fermentation are shown in Figure 2. Among the fermented sample using juice mixture and C1 (fermented coffee without juice mixture), 10B (10°Brix) showed the highest antioxidant properties after 48 hours of fermentation (DPPH radical-scavenging 2003.77 µmol TE/g DW, total polyphenols content (TPC) 132.35 mg GAE/g DW, and total flavonoid content (TFC) 433.78 mg QE/g DW). This is because the optimization of growth conditions for *S. cerevisiae* and *B. subtilis* were reported at around 10°Brix [15], [29]. At this condition, they may strong active to provide some organic compounds which might be associated with proteolytic enzymes that hydrolyze the complexes of phenolics into simple molecules which make them more bioavailable [17], [29]. At lower sugar concentration samples, there were not enough nutrients for the growth of microorganisms as well as support for producing organic compounds in fermentation. By contrast, at higher sugar concentration samples, there may occur osmotic pressure due to the amount of sugar in a higher medium which caused to breakdown of the microorganism cell wall and decreased number of microorganisms in the fermentation medium. Therefore, fermentation quality was low [15]. However, when comparing those to C (origin green coffee beans), there were no significant differences between them. This is because the time of fermentation for 48h was not the best condition for this coffee fermentation. Therefore, the fermentation time in this experiment was not proper, making the result lower than the unfermented green coffee beans. The antioxidant properties value for C and C1 (DPPH radical-scavenging 2453.54, 1534.93 µmol TE/g DW, TPC 138.7, 81.90 mg GAE/g DW, and TFC 425.15, 311.02 mg QE/g DW, respectively). In comparison between the two control samples, the antioxidant activity value of C1 was significantly lower than those of the C ( $P < 0.05$ ). This was because the soluble antioxidant and phenolic compounds in green coffee beans might be eluted into the medium.

In summary, coffee fermentation by using juice mixture at different concentrations had a positive effect on the antioxidant properties of coffee beans, especially in total flavonoid content. This is similar to the results of soaking green coffee beans in the mulberry extract [7].



**Figure 2.** Effect of concentration of juice (Brix degree) on antioxidant properties of green coffee beans. Antioxidant activity (A), total polyphenol content (B), and total flavonoid content (C) of fermented coffee extracts.

\*Different letters above the bars indicate a statistically significant difference at  $p < 0.05$  among treatments.

\*\*C, unfermented coffee; C1, fermented coffee without juice mixtures; 5B, fermented coffee at 5°Brix; 10B, fermented coffee at 10°Brix; 15B, fermented coffee at 15°Brix.

#### 4. Conclusions

In conclusion, this study was carried out to find the best fermentation condition of fermentation time and amount of juice through Brix degree that related to the antioxidant properties of coffee beans. As the result, there was not a significant difference in terms of antioxidant properties between fermented and original coffee beans. Antioxidant properties dramatically reduced during the early stages of fermentation, but it was shown to be able to overcome it through this study. It could be observed that the time (48 hours) of fermentation in 10°Brix, and  $10^7$  cells/mL at room temperature should be selected as the optimal condition in the fermentation of green coffee beans. Further study should be focused on other fermentation factors such as fermentation time, microorganism population, the ratio of microbes, the ratio of juices, fermentation temperature, acidity, as well as type of enzymes created during fermentation.

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**Dinh Yen Nhi** ungraduated at Food Technology Department at International University \_ Vietnam National University, Ho Chi Minh City. She is the author of two papers related to the effect of coffee processing to antioxidant properties and sensory profile; application of the digestive bioprocessing model on coffee fermentation. She is teaching assistance for Enzyme and Food Fermentation, Technology of Coffee, Tea, and Cocoa classes. She has supported the coffee flavor research.



**Nguyen Quang Duy** is a lecturer at the Department of Food Technology, Faculty of Chemical and Food Technology, Ho Chi Minh City University of Technology and Education (HCMUTE), Vietnam. He received his Bachelor of Engineering and Master of Engineering by research from International University – Vietnam National University in Ho Chi Minh City in 2016 and 2019, respectively. His background focuses on microbial enzymes and their application on industrial plants, such as coffee, tea and cocoa. The researches aim to increase nutritional and added value to those products as well as innovate new food products in the market. He is the author and co-author of more than ten scientific papers which published in the international as well as national journals. He has experience in doing research, conducting projects and especially in technology transfer from 2014 until now.



**Le Hong Phu** currently works at International University \_ Vietnam National University Ho Chi Minh City as a role as an associate professor for Food Technology Field. He received his bachelor's degree in biology from University of Science \_ Vietnam National University, Ho Chi Minh City in 2000. He received his master and Ph. D degrees in Biochemistry from University of Science \_ Vietnam National University, Ho Chi Minh City in 2003, 2012, respectively. He was promoted to associate professor in 2017. His research areas cover applied biochemistry, applied microbiology, as well as functional food. He is the author of nearly 50 international and national articles and the leader of 4 transfer research projects. He has successfully guided more than 100 students and graduates in Food Technology and Biotechnology. The articles are focused on objects such as coffee, pepper, cocoa, and others such as ginseng and algae that are being cultivated in the central provinces and the Central Highlands in Vietnam. Other research is aimed at producing convenient functional products that can be used immediately, such as instant tea. He focuses on research on fermentation conditions for the production of fermented coffee with microbes, using a combination of enzymes to process the extraction with high efficiency, as well as peel off the skins in the production of white pepper, honey coffees.