E-ISSN: 2655-8777

3Bio

JOURNAL OF BIOLOGICAL SCIENCE, TECHNOLOGY AND MANAGEMENT

Volume 4, No 1, 2022

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School of Life Sciences and Technology
Institut Teknologi Bandung - Indonesia

3BIO: Journal of Biological Science, Technology and Management

Volume 4 • Number 1 • 2022

This journal and individual articles are published at http://journals.itb.ac.id/index.php/3bio
a series imprint of the ITB Journal – an open access publisher.

ISSN 2655-8777 (Online)

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Effects of Leaf Storage and Distillation Time on the Quality of Eucalyptus (*Eucalyptus grandis*) Essential Oil

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Received: 2022-01-30
Accepted for publication: 2022-04-03

Abstract

Essential oils such as eucalyptus oil are widely used as antioxidants, antimicrobial, anti-inflammatory, fragrance, and other medical needs. This oil is extracted from eucalyptus terpenoid compounds, e.g., cineole. This study aims to evaluate the effect of the leaf storage time and distillation time (DT) on *Eucalyptus grandis* essential oil yield and quality. Three DT treatments (i.e., 2 h, 3 h, and 4 h) and storage time of eucalyptus leaf in warehouse (i.e., 2 h, 3 h, and 4 h) were applied on eucalyptus leaf samples. The quality of the eucalyptus essential oil was determined using cineole content, solubility in alcohol, and optical rotation. The essential oil yield ranges between 0.165-0.220%, with the maximum yield of 0.220% on 3 days of storage and 4-hour distillation time. The cineole content ranged between 10-30% and the highest at 3 h DT with a storage time of 2 days. The optical rotation on the tested specimen showed that the index of refraction produced in oil projections was positive. The samples in all treatments showed high solubility in alcohol. This study showed that storage time of 3 days and 3-hour distillation time resulted in the most optimal eucalyptus essential oil yield, and the excellent quality of oil produced has a high potential for health product applications.

Keywords: Eucalyptus oil, distillation, storage time, *Eucalyptus grandis*, 1,8 cineole

1. Introduction

Essential oils are organic chemicals extracted from plant parts such as leaves, twigs, wood, roots, and barks (1). One of the essential oil characteristics is the presence of various essences or volatile compounds with beneficial properties such as antioxidants (2), antibacterial (3), and anti-inflammatory (4). The development of the essential oil industry started in the early 20th century due to its wide use, making them one of the most important processed products (5).

Data published by Statistics Indonesia in 2020 mentioned that the Indonesian export rate of essential oil has increased during the last three years compared to the 3122,1 tons(x1000) of essential oil export in 2017 and 4819,7 tons(x1000) in 2020 (6). This data suggests that the production process may be ineffective for several reasons, e.g., the yield obtained is too small, lack of management, or less competitive oil quality. As the essential oil is strongly bound to plant cells, the dry weight will have a higher yield value.

Essential oils are produced by isolating the plant's natural ingredients by extracting compounds in the deepest cell layers in the leaves, flowers, twigs, stems, and roots (7). Eucalyptus essential oil is extracted from its leaves and twigs using the wet distillation process (8), steam (1), and other extraction methods. A study by Ratnaningsih & Insusanty (9) found that the eucalyptus refining process produced a maximum oil yield of 0.45%. Eucalyptus leaf distillation is affected by environmental factors such as humidity, temperature (10), leaf conditions, and distillation time (11).

Sugihmukti village, Ciwidey, West Java, is a potential source of abundant eucalyptus leaf. Using eucalyptus leaf from this village as a natural resource for eucalyptus oil production will support the village income and development. This idea aligns with the concept of regional development related to sustainable natural resources (12). This study focused on improving production effectiveness by increasing eucalyptus oil yield by modifying the duration of leaf storage and distillation time. Eucalyptus

DOI: 10.5614/3bio.2022.4.1.1

leaf storage determines the quality of the post-harvested leaves in the storage process (13). The distillation period determines the amount of eucalyptus oil yielded from the distillation.

Results obtained from this research can serve as a reference for eucalyptus oil production yield and quality optimization that can contribute to future regional potential development. Therefore, it is necessary to adjust the results according to SNI 8834:2019 as adopted ISO 770:2002. This process is important so that aspects of production can be applied further in the next process.

2. Methodology

2.1. Sample Preparation

Eucalyptus (*Eucalyptus grandis*) leaves used to distillate eucalyptus oil were taken from the forestry plantation as PERHUTANI's forest at Sugihmukti village, Ciwidey, West Java. The weight of eucalyptus leaves used per distillation process was 8 kg. Treatments applied were the variations of storage time (i.e., 1, 2, and 3 days) and distillation time (i.e., 2, 3, and 4 hours). The distillation method used in this study was steam distillation with a specification temperature of 105±2℃, air pressure of ±1200 m above sea level, and air temperature is 14-20℃. The steam distillation scheme is presented in Figure 1.

The complete variations of treatment are presented in Table 1. The variations in this study were used to determine the distillation process for each sample. The sample code uses the notation A as leaf storage time, B as distillation time, and numbers as a marker of the time used.

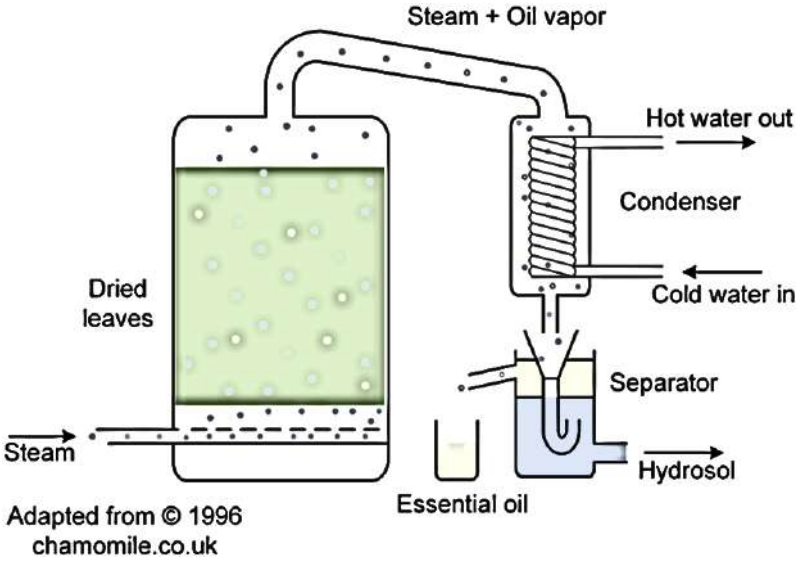

Figure 1. Steam Distillation Scheme for distillate eucalyptus leaf (14)

Table 1. Research Variations

Number	Sample code	Treatment	
		Storage time (day)	Distillation time (hours)
1	A1B1	1	2
2	A1B2		3
3	A1B3		4
4	A2B1	2	2
5	A2B2		3
6	A2B3		4
7	A3B1	3	2
8	A3B2		3
9	A3B3		4

DOI: 10.5614/3bio.2022.4.1.1

2.2. Sample Test

The essential oil obtained was subjected to several tests to determine its quality. All tests were done at the Laboratory in Indonesian Medicinal and Aromatic Crops Research Institute (IMACRI), Bogor, West Java, Indonesia. Selected tests were, i.e.,

1. Total Yield of Eucalyptus Oils

Determination of total eucalyptus oil yield using the following equation :

$$Yield\ (\%) = \frac{Mass\ of\ essential\ oil\ (g)}{Mass\ of\ eucalyptus\ leaf\ (g)} \times 100\% \quad (1)$$

This determination is used to determine the percentage of oil produced from eucalyptus leaves used at each storage time.

2. 1,8 Cineole content test

The cineole content test was done to determine the cineole content in the essential oil. The cineol content is an indicator of antioxidants in eucalyptus oil. Cineole/1,8 cineole structure is the same as in Figure 2. This test complied with the Indonesian standard SNI 8834:2019 about Eucalyptus essential oil (*Eucalyptus globulus* Labill.) rectified (ISO 770:2002, MOD) (15).

3. Optical Rotation Test

The optical rotation test was carried out to determine the purity level of the essential oil obtained. Optical rotation is the index of refraction produced in oil projections when exposed to light. This test complied to the Indonesian standard SNI 8834:2019 about Eucalyptus essential oil (*Eucalyptus globulus Labill.*) rectified (ISO 770:2002, MOD) (15).

4. Solubility Test in Alcohol

This test was conducted to determine the essential oil level of release in alcohol. This solution is used as a reference in the further processing step. This test complied with the Indonesian standard SNI 8834:2019 about Eucalyptus essential oil (*Eucalyptus globulus Labill.*) rectified (ISO 770:2002, MOD) (15).

3. Results and discussion

3.1. Eucalyptus Leaf Oil Yield

Results showed that the extraction yield in every storage time treatment increased with the increase in the duration of distillation (Figure 3). The longer the distillation time, the higher the yield produced in each storage time treatment.

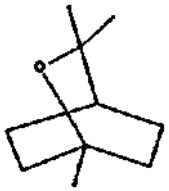

Figure 2. 1,8 Cineole structure (Eucalyptol)

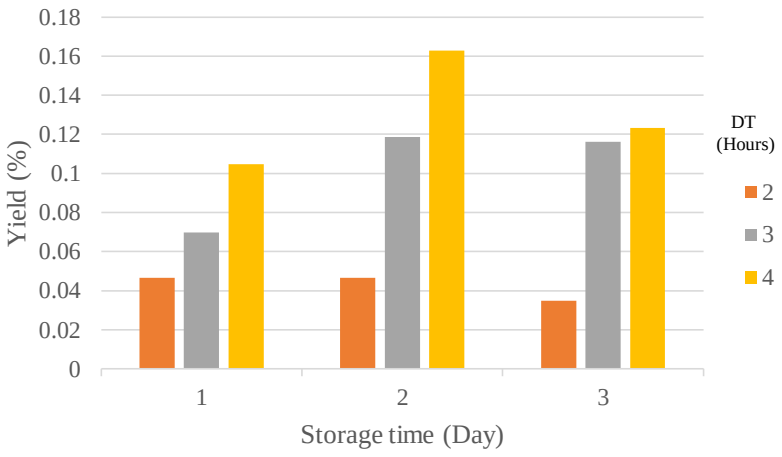

Figure 3. Eucalyptus leaf oil sample yield in different treatments

DOI: 10.5614/3bio.2022.4.1.1

The storage time treatment results showed the highest average yield of 2 days, followed by 3 days and 1 day. The highest yield value of 0.168% was obtained from sample A2B3 with a storage time of 2 days and a distillation time of 4 hours. The yield value of the 2-hour distillation sample at all storage times ranges from 0.03-0.06%, with increasing sample yield values of 0.047% (A1B1), 0.047% (A2B1), and 0.035% (A1B1). The yield value of the 3-hour distillation sample at all storage times ranges from 0.06-0.12%, with increasing values of 0.069% (A1B2), 0.119% (A2B2), and 0.116% (A3B2). In comparison, the 4-hour distillation yield ranges from 0.1-0.17% with increasing values of 0.105% (A1B3), 0.163% (A2B3), and 0.123% (A3B3).

The measured eucalyptus leaf oil sample showed that the leaf storage time could increase the amount of yield. The process of leaf storage causes degradation of the leaf cell structure, making it easier to isolate the oil (16). However, a more extended storage period will cause continuous cell damage, which adds possibility of oil evaporation that will eventually decrease the yield (17). Indeed, in our study, we found the highest yield on day 2, and the yield slightly decreased on day 3. But, another study by Ratnaningsih & Insusanty (9) showed an optimum *eucalyptus pellita* leaf storage time of 3 days.

Another treatment showed that the longer the distillation time resulted in a higher yield. The highest yield was demonstrated by 4 hours of DT followed by 3 and 2 hours. A possible explanation is that a relatively large energy/steam propulsion is needed to extract oil from the eucalyptus leaf cell. The results also found that the distillation time of 2 hours to 3 hours experienced the most drastic increase compared to 3 to 4 hours (13). The longer distillation will increase the amount of yield, but there will be a culmination time point that causes no increase in the amount (1). These results follow a study by Ratnaningsih & Insusanty (9), which shows that the distillation of *eucalyptus pellita* leaf oil produces a maximum yield at 5-6 hours.

3.2. The concentration of 1.8 cineole eucalyptus leaf oil

The measurements of cineole levels in eucalyptus leaf oil showed that cineole levels fluctuated with storage and distillation times. The value tends to decrease based on the storage time of eucalyptus leaves (Figure 4).

The highest cineole levels were shown in samples A1B3 (25.83%) and A2B1 (26.65%). Meanwhile, the lowest cineole content was shown by sample A3B1 (13.75%). The cineole content value indicates cineole in every ml of eucalyptus leaf oil. Cineole is an antioxidant compound that will depend on the conditions of the sample and the environment. The measurement results found a decreasing trend of cineole content with the increase in the storage time from day 1 to day 3. The cineole content ranged from 10-30% on the 1st to 3rd day of storage. When compared with SNI 8834:2019 or the adoption of ISO 770:2002, this value is lower. The standard value in SNI is >40%. But the research of Ahmad et al. (13) showed that the content of 1.8 cineole ranged from 18-75% due to external variables such as temperature, altitude, and soil type.

Apart from that, 1,8 Cineole as an antioxidant compound can be easily oxidized due to environmental influences (17). The day 1 sample has the highest cineole compared to other samples because the leaves are fresher. The fresh sample has good and potent antioxidants conditions; thus, the antioxidants detected are higher than the withered leaves. The cineole content on the 2nd and 3rd days of storage decreased because the Eucalyptus leaves used were wilted and damaged due to the storage process. Meanwhile, distillation time does not affect the cineole content of eucalyptus leaves. On day 1 samples, the longer the distillation resulted in a higher cineole content. On the contrary, day 2 and 3 samples showed decreased cineole values with increased storage and distillation time. The cineole levels found in this study follow the results of Maail & Purimahua (18), which show damage to antioxidants due to environmental degradation when not appropriately preserved (18).

3.3. Solubility in alcohol

The measurement of the solubility of eucalyptus leaf oil showed that all eucalyptus samples were soluble in alcohol (Table 2). The ratio used was 1:1 to 1:7.

All eucalyptus leaf oil samples were soluble in alcohol in a ratio of 1:1 to 1:7, indicating that eucalyptus leaf oil is easily dissolved in alcohol. The ability to be soluble in alcohol means that eucalyptus leaf oil contains terpenoid compounds such as 1,8 cineole (13).

Indeed, the samples of eucalyptus oils (Figure 5) showed a 15-30% cineole content. The alcohol solubility ratio of 1:1 to 1:7 alcohol shows that the quality of extracted eucalyptus oil fulfills the national standard of SNI 8834:2019 (9). If this data compares with SNI 8834:2019, the result shows that all treatment has good quality. But, if we will use eucalyptus oil for industry and need to reduce the cost of production, maybe sample A2B2 has a good chance. The solubility in alcohol 1:1 shows that eucalyptus oil only needs 1 part alcohol to dissolve 1 part eucalyptus oil.

DOI: 10.5614/3bio.2022.4.1.1

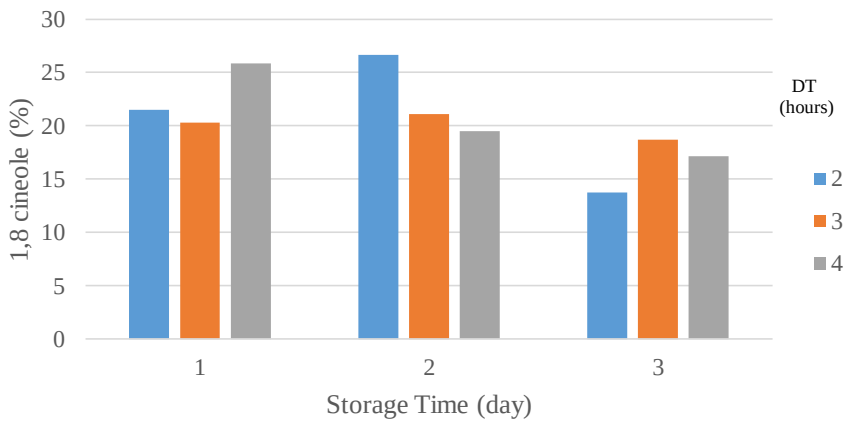

Figure 4. 1,8 cineole content of Eucalyptus oil

Table 2. The solubility of Eucalyptus leaf oil in alcohol

No	Sample code	Solubility in alcohol	Solubility in alcohol SNI 8834:2019
1	A1B1	Soluble (1:4)	Min 1, Max 7
2	A1B2	Soluble (1:6)	
3	A1B3	Soluble (1:4)	
4	A2B1	Soluble (1:2)	
5	A2B2	Soluble (1:1)	
6	A2B3	Soluble (1:7)	
7	A3B1	Soluble (1:7)	
8	A3B2	Soluble (1:5)	
9	A3B3	Soluble (1:5)	

Figure 5. Samples of eucalyptus oil

DOI: 10.5614/3bio.2022.4.1.1

3.4. Optical Rotation Index

The optical rotation index measurement of eucalyptus oil samples showed a value range of 20-30̊ (Table 3). The optical rotation index value determines the purity of eucalyptus leaf oil.

From nine samples tested, only three detected optical rotation values. Those three samples showed more than 20 degrees rotation value, with the highest value found in A3B1 (24.75), followed by A3B2 (22.55) and A3B3 (22.50̊). Many essential oils, including eucalyptus oil, contain fragrant chiral compounds that rotate levorotatory and dextrorotatory plane-polarized light. The measurement of the optical rotation of the essential oil shows the diversity of optically active compounds in natural compounds. The optical rotation value indicates the purity of a specific compound, and the positive value indicates that the molecular structure has a rotation to the right (10). Compared with the result of other studies with an optical range of +2 to +8, this study indicates a different supporting structure of eucalyptus oil or eucalyptus oil concentration and temperature higher than standard (18). The net rotation, of course, depends on all the chiral compounds present and their relative quantities. There is no simple direct correlation between the net rotations of the experimental and standard essential oils in Table 3. The exact amounts of the different compounds may vary depending on the season, variety and origin and possibly optical rotation as well (19). In another way, the optical rotation exhibited by a chiral medium depends on the optical path length, the wavelength of the light used, the temperature of the system, and the concentration of dissymmetric analyte molecules (20).

Table 3. The optical rotation index of eucalyptus leaf oil samples

No	Sample code	Optical Rotation (̊)	Optical Rotation in SNI 8834:2019
1	A1B1	NA	+2, +8
2	A1B2	NA	
3	A1B3	NA	
4	A2B1	NA	
5	A2B2	NA	
6	A2B3	NA	
7	A3B1	24.75	
8	A3B2	22.55	
9	A3B3	22.5	

*notes : NA (not aviable)

In this study, the extraction process focused on Eucalyptus's leaves and twigs because the oil part tends to be on the leaves and twigs, although there is a yield of essential oil in other parts. Marques et al. (7) showed that the amount of essential oil could be obtained from several parts such as leaves, twigs, wood, and roots (9). However, this research is fair because the objective is to utilize the wasted leaves. In general, the extraction process used in this study allows it to be used on other species of eucalyptus leaves, as has been done by Marques et al. (7) in a variety of *Eucalyptus globulus*. These results can occur because the family of Eucalyptus has similarities in terms of the content of 1,8 cineole compounds (7). This study allows the method to be used more widely for other varieties. So that use on a larger scale can be increased by farmers or eucalyptus landowners.

From the production aspect, the optimum storage time is 2 days. However, if viewed from research by Marques et al. (7) that shows maximum yield in dry leaf conditions, this extraction process can be further improved. In addition, the production process will pay attention to the distance between the plantation and the production area, temperature, humidity, and air pressure (13). So that in the process of sustainability and minimizing bias in the dry and wet seasons, further studies are needed regarding the use of dry eucalyptus leaf species.

DOI: 10.5614/3bio.2022.4.1.1

4. Conclusion

This study concludes that eucalyptus oil produced from the refining process has a maximum yield of 0.165% with a content of 1.8 cineole at 20-30%. The storage process affects the quality of eucalyptus oil in terms of the content of 1,8 cineole. Meanwhile, the length of distillation time resulted in differences in yield produced. To reach the maximum yield, the distillation time needed was 4 hours. Eucalyptus leaf oil has good solubility in alcohol from a 1:1 to 1:7 ratio. The quality of eucalyptus leaf oil is also indicated by its optical rotation value. However, this study requires further refinement. More sample replication is needed to test results statistically. By comparing the yield and quality of the eucalyptus oil obtained, eucalyptus oil production can be improved. In addition, oil obtained from different parts of the eucalyptus tree can be an interesting study subject. These processes and evaluations will support a more comprehensive study on eucalyptus oil production.

Acknowledgements

The authors thanked PT. Geo Dipa Unit Patuha, Indonesia, for providing *Corporate Social Responsibility* (CSR) 2021. The authors also thanked the Faculty of Industrial Technology, Institut Teknologi Bandung, for providing the necessary facilities for testing.

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DOI: 10.5614/3bio.2022.4.1.1

e-ISSN: 2655-8777 http://journals.itb.ac.id/index.php/3bio

Estimation of Minimum Viable Population of Long-tailed Macaques (*Macaca fascicularis* Raffles 1821) in Support of Wildlife Management in Ir. H. Djuanda Grand Forest Park

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Received: 2022-01-07
Accepted for publication: 2022-03-22

Abstract

Most long-tailed macaques (LTMs) in Southeast Asia are commensal animals, which means they can coexist and benefit from humans. This interaction potentially triggers disturbance and disease transmission caused by LTMs, for instance, in Ir. Djuanda Grand Forest Park (GFP). A previous study indicates an overpopulation of LTMs in this area. Population control of this macaque requires appropriate ecological considerations, including determining the minimum viable population (MVP). This study aims to estimate the MVP of LTMs in Ir. Djuanda GFP area is the key input for its population control scheme. The MVP is determined based on population growth simulation using the Lefkovitch matrix. In combination with the estimate of population growth, the MVP was used to formulate the population control scheme of LTMs, by considering the current wildlife management regulations and the perception of Ir. Djuanda GFP area manager obtained through interviews. This study found six groups of LTMs in Ir. Djuanda GFP with a total population of 229 individuals, a sex ratio of 1:1.05, an age class ratio of infants, juveniles, sub-adults, and adults of 16.6%:25.7%:32,3%:25.3%, and a population growth rate of 19.7%/year. The estimate of MVP of this primate is 230 individuals, with an average group size of 38 individuals and an age class ratio of infants, juveniles, sub-adults, and adults 30%:9.1%:27.4%:23.5%. Based on wildlife management regulations and area manager perceptions, population control schemes can be implemented for relocation and captive purposes. The population control scheme consists of an age-class control scheme and a group control scheme. The age-class control scheme controls the population by 334 individuals, while the colony control scheme controls 406 individuals.

Keywords: Human-animal conflict, Population control, Nature Conservation Area, Population Ecology

1. Introduction

The long-tailed macaque (*Macaca fasscicularis*, hereafter LTMs) is a widely distributed primate in Java [1]. This animal can live in various habitats, from the coastal area to primary forests. In Southeast Asia, mostly LTMs are commensal animals (i.e., animals that live with and benefit from humans), and few are still living wild and undisturbed by humans [2]. The human activity area provided new food sources for LTMs, such as fruit, food scrap, and garbage. When food sources are concentrated in an area, these animals will tend to be concentrated in that food source area, and there is an overlap between human activity areas [3]. In several locations in Indonesia, these animals live overlapping areas of human activity, such as in Pangandaran Nature Reserve and Nature Park, Kreo Cave Tourism Area in Semarang, Grojogan Sewu Nature Tourism Park, Tawangmangu Karanganyar, and several other natural attractions area [4].

Commensal conditions cause interactions that occur between humans and LTMs. This interaction can cause various impacts given and received by each party. Areas of human activity provide more food sources and become a dietary supplement for LTMs. In addition, LTMs will be more active around the area to minimize travel costs and maximize forage exploration [5]. However, this LTMs behavior causes losses to humans because there are mostly damaged crops and disturb the community in the

DOI: 10.5614/3bio.2022.4.1.2

settlement area [6]. LTMs provide a distraction by chasing, grabbing, and attacking for food in nature tourism areas. Their behavior is because LTMs are accustomed to being given food by visitors, causing them to adapt and know that humans bring the food they want [7].

LTMs population size influences the intensity of the disturbance. As the population size increases, the frequency of tension, fighting, and aggression within and between groups will increase. Some groups will increasingly disturb visitors to ask for food, or leave the area to damage agricultural land and plantations, also settlement areas [8]. On the other hand, LTMs are an intermediary for transmitting several diseases such as SFV (Simian Foamy Virus), Herpes B, Flu A and B, and Measles through direct contact [9]. This condition causes disturbances that occur not only to provide material losses to humans but also in the form of disease transmission carried by these animals.

One area that has interaction between these two parties is Ir. H. Djuanda Grand Forest Park (hereafter GFP). Ir. H Juanda GFP is a nature conservation area that is also used as a nature tourism area. The tourism activities that occur here present interactions between visitors and LTMs. In addition, the land around the area consists of fields and residential areas. These two conditions attract LTMs and cause wildlife disturbance. For example, in 2019 LTMs damaged settlements and crops areas located in Mekarwangi Village, Lembang District, which is located on the Ir. H. Djuanda GFP border. On the inside of the area, especially on weekends, tourist activity becomes very crowded, especially along the main track that divides the area. This causes LTMs to come to the crowd to get food brought by visitors by asking for or even pursuing and stealing. Furthermore, based on data collection conducted in 2020, it is known that the total population of LTM in this area is around 376 individuals, while the carrying capacity of the area is only 129 individuals [10]. This causes an increase in the intensity of disturbances that occur inside and outside the area by LTMs.

To minimize potential disturbances occurring at Ir. H. Djuanda GFP, it is necessary to carry out population control measures. Population control can be done in the form of relocation and harvesting activities to balance the population size in its natural ecosystem. Harvesting actions that will be carried out need to consider policies on the use of wildlife, the sustainability of the remaining population, and the legal status and protection of the animals whose populations will be controlled [11].

Population control measures require specific references to maintain the existence of the remaining population in the area. One of the references that can be used is the size of the minimum viable population (MVP). MVP is the smallest measure of an isolated population in a particular habitat, which has a 99% chance of survival for 100 years amid various disaster risks caused by certain factors [11]. In determining the MVP, demographic parameter data and growth population models are needed to estimate the number of individuals from LTMs utilized and ensure the remaining population remains stable in the future [12]. This study aims to determine the MVP size of the LTMs in the Ir. Djuanda GFP. The MVP will then be used to determine the population control scheme by considering the dynamic conditions of the LTMs population in the area.

2. Methodology

2.1. Time and Location

This research was conducted from April to December 2021 in Ir. H. Djuanda GFP Area, Jl. Ir. H. Djuanda No.99, Ciburial, Cimenyan District, Bandung City, West Java (Figure 1). This area has an altitude of 770 - 1330 meters above sea level, with a basin-shaped landscape from the Cikapundung river flanked by two steep slopes. Based on the climate classification of Ferguson and Schmidt (1951), this area has a B climate type (9 wet months, 3 dry months) and a type C climate (8 wet months, 4 dry months). The land cover in this area is secondary dryland forest, with dominant species such as *Pinus merkusii*, *Caliandra callothrysus*, *Swietenia sp.*, and *Bambusa sp* [13]. This research was conducted in three stages, i.e. preliminary survey, data collection on LTMs population dynamics, and data analysis. The preliminary survey was carried out from April to August 2021, while data collection on LTM encounters was carried out from September to October 2021.

2.2. Data collection

Primary data consists of information on population dynamics of LTMs and the perception of Ir. H. Djuanda GFP area manager related to LTMs issues. Secondary data consists of various regulations related to the management and use of wild animals.

Population dynamics data were obtained through the collection of LTMs population demographic data. Population demographics were obtained through field observations using the point count method at 6 points in the area (Figure 2) [14]. The method of counting points selected topographical characters that do not allow the transect method. On the other hand, the selection of observation points was carried out purposively, referring to the preliminary survey results. The selected point is the point most visited by LTMs, but not visited by the same group. Observations were carried out in the morning (8 – 11 am) and afternoon (1– 4 pm), which is 2 days at each point and takes the maximum recorded group size. The data collected

DOI: 10.5614/3bio.2022.4.1.2

were the number of individuals grouped by sex and age class. Age class grouping data collection was carried out qualitatively, considering the difficulty of determining the age of individual animals in the wild and since no historical data on the population of LTMs owned by the manager. Age classes are grouped as infants (0-1.5 years), juveniles (1.5 – 4 years), sub-adults (4 – 9 years), and adults (9 – 21 years) [15]. The population demographic data were used to observe the LTMs population's growth and determine the MVP size of each age class and colony.

The perception of Ir. H. Djuanda GFP manager was obtained through interviews that were conducted in a semi-structured method. The information collected included opinions of area managers regarding LTMs problems, such as causes of disturbance and overpopulation, losses received, and LTMs management actions.

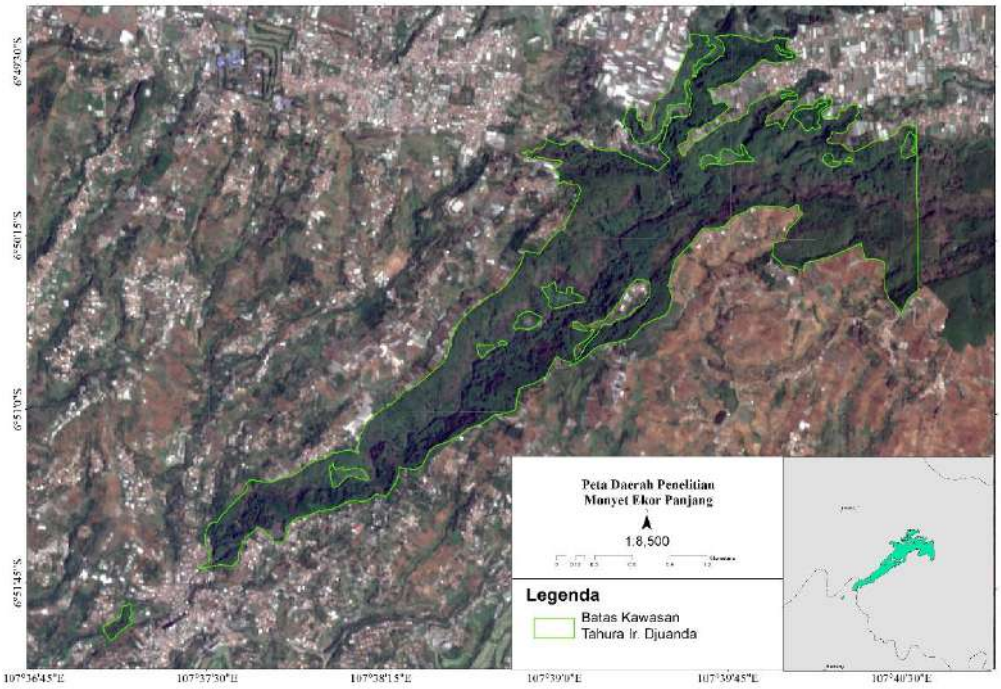

Figure 1. Research Study Area

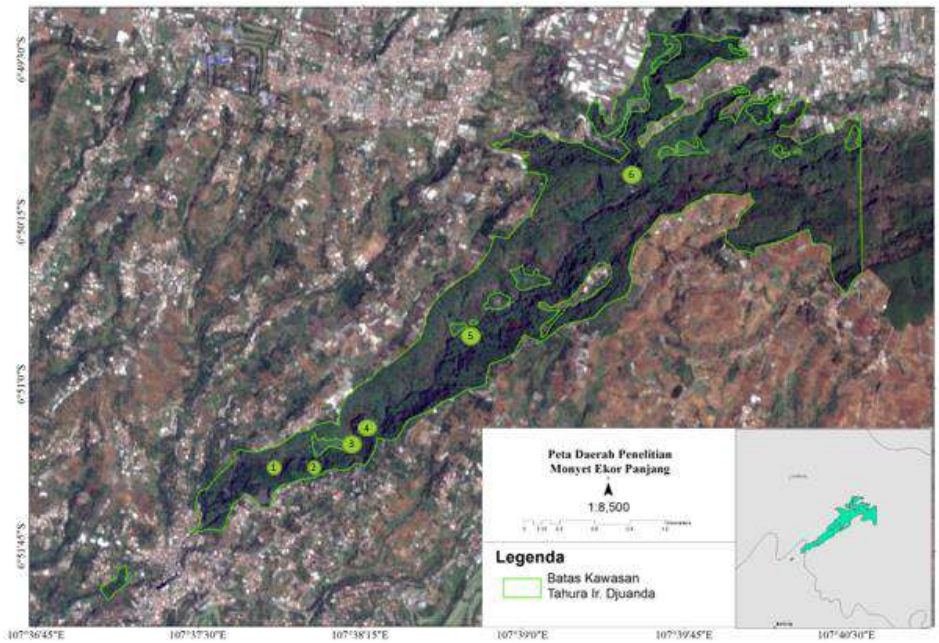

Figure 2. The observation points of LTMs

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2.3. Data Analysis

2.3.1.Long-tailed Macaques's Dynamics Population

LTMs population growth was analyzed by using Lefkovitch Matrix. The Lefkovitch matrix is a growth model developed from the Leslie matrix. The Leslie matrix is a growth model that uses a discrete age structure. However, it will be challenging to determine the individuals' age in population studies that are not using cohort (observations are made from birth to death of all populations), such as those carried out in nature. The Lefkovitch matrix is an advanced development model that refers to the stage-class of an individual, so it can be used on species of unknown age as long as the life stage can be determined qualitatively [16].

The assumptions used in the Lefkovitch model include:

1. There was no population migration during the observation
2. The population growth model is density-independent
3. LTMs are classified as a birth flow model, where births can occur every year because these animals do not have a mating season.

This matrix was simulated in a Microsoft Excel application. The simulation duration used was 25 years, referring to the maximum age of LTMs in the wild. The form of the Lefkovitch matrix is a simple transition matrix that is formulated as follows

$$M_L \times N_t = N_{t+1}$$

$$\begin{bmatrix} \delta_I & 0 & F_{SA} & F_A \\ Px_I & \delta_J & 0 & 0 \\ 0 & Px_J & \delta_{SA} & 0 \\ 0 & 0 & Px_{SA} & \delta_A \end{bmatrix} \times \begin{bmatrix} X_{I,t} \\ X_{J,t} \\ X_{SA,t} \\ X_{A,t} \end{bmatrix} = \begin{bmatrix} X_{I,t+1} \\ X_{J,t+1} \\ X_{SA,t+1} \\ X_{A,t+1} \end{bmatrix}$$

F_{SA} = Sub-adult class fecundity
F_A = Adult class fecundity
Px_I = Survival probability of infant class
Px_J = Survival probability of juvenile class
Px_{SA} = Survival probability of sub-adult class
δ_I = Proportion of remaining infant class
δ_J = Proportion of remaining juvenile class
δ_{SA} = Proportion of remaining sub-adult class
δ_A = Proportion of remaining adult class, with a birth age limit of 12 years
$X_{I,t}$ = Number of infant class when t-years
$X_{J,t}$ = Number of juveniles class when t-years
$X_{SA,t}$ = Number of sub-adult class when t-years
$X_{A,t}$ = Number of adult class when t-years, with a birth age limit of 12 years
$X_{I,t+1}$ = Number of infant class when t+1 years
$X_{J,t+1}$ = Number of juveniles class when t+1 -years
$X_{SA,t+1}$ = Number of sub-adult class when t+1-years
$X_{A,t+1}$ = Number of sub-adult class when t+1 years, with a birth age limit of 12 years

2.3.2.Estimation of Minimum Viable Population Size

MVP size refers to the basic condition $N_0 = N_t$ [17]. Population N_o contains information related to the ability to increase and decrease the population in each age class, such as the number of births produced by productive female groups and deaths in each age class (equation i). The N_T population describes the population growth derived from the Lefkovitch matrix, which occurs during the period of t-years (equation ii). MVP value of each age class can be determined by finding the intersection between the population N_0 and N_T. The algebraic calculations performed can be seen in the following formulation:

Equation (i):

$$0 = (F_{SA} \times X_{SA}) + (F_A \times X_A) - ((M_A \times X_A) + (M_{SA} \times X_{SA}) + (M_J \times X_J) + (M_I \times X_I))$$

Equation (ii):

$$0 = N_t - ((F_{SA} \times X_{SA} + F_A \times X_A) + (Px_I \times X_I + Px_J \times X_J + Px_{SA} \times X_{SA}) + (\delta x_I \times X_I + \delta x_J \times X_J + \delta x_{SA} \times X_{SA} + \delta x_A \times X_A))$$

Since equations (iii) and (iv) are comparable,

$$(F_{SA} \times X_{SA}) + (F_A \times X_A) - ((M_A \times X_A) + (M_{SA} \times X_{SA}) + (M_J \times X_J) + (M_I \times X_I)) =$$

$$N_t - ((F_{SA} \times X_{SA} + F_A \times X_A) + (Px_I \times X_I + Px_J \times X_J + Px_{SA} \times X_{SA}) + (\delta x_I \times X_I + \delta x_J \times X_J + \delta x_{SA} \times X_{SA} + \delta x_A \times X_A))$$

2.3.3.Formulation of Population Control Scheme

The population control scheme was formulated based on considerations obtained from the study of regulations on the management and use of wildlife, the perspective of Ir. H. Djuanda GFP manager, the ability of population growth, and the MVP. Regulatory considerations and managers' perspectives will later produce alternative objectives for population control activities. Furthermore, the scheme of population control was reviewed through the effect of applying MVP by age class (control is only carried out on age classes that have a size above the age class MVP) and groups (control will be carried out when all age class sizes in a group have passed the MVP) on population growth.

This study also considered the current regulation on the use of wildlife. In addition, this study analyzed the potential

DOI: 10.5614/3bio.2022.4.1.2

costs and revenues of LTMs population control and additional recommendations to overcome the problem of LTMs in the Ir. H. Djuanda GFP area.

3. Results and discussion

3.1. Long-tailed Macaques' Population Dynamics

Population Structure

This study found that each LTM group in six observation points has a different population structure, both in terms of age class and gender (Table 1). The total population from all observation points is 229 individuals, with an average of 38 individuals/point. Each point has 1 group because at each point it is known to have one group due to rejection and fights by the group that has occupied a point against the new group that has just arrived.

The population size of LTMs varies based on the type of habitat. The primary forest in Peucang Island, Ujung Kulon National Park, has an average LTM population of 21 individuals/group [12]. In Lampung Province, the average LTM population is 20 individuals/group in primary forest, 39 individuals/group in secondary forest, 32 individuals/group in coastal forest, and 47 individuals/group in mixed farmland [18]. Compared to the habitat type of Ir. H. Djuanda GFP, i.e., secondary dryland forest, the LTMs population in Lampung Province is quite similar to the one in Ir. H. Djuanda GFP. This condition happened because they have the same land cover type. Furthermore, it is not stated with certainty whether the study area in the province of Lampung has the same conditions for natural and human activities as that of Ir. H. Djuanda GFP. In addition, the population size of a group is also influenced by the rate of birth, death, immigration, emigration, ability to compete with other groups, and the process of group formation [19].

Sex Ratio

Table 2 presents the sex ratio of the LTMs group in Ir. H. Djuanda GFP, which ranges from 1:1 to 1:1.3 on each point. The sex ratio of the total LTMs population is 1:1.1, where the number of male and female individuals is almost the same. A similar condition was also found in other studies, such as in Tenau Monkey Cave Recreation Park (TRGMT) Kupang, with an LTMs sex ratio of 1: 1,285 [20]. The population of LTMs in Peucang Island also had a similar sex ratio, i.e., 1:1,2 [12]. The sex ratio influences the level of competition in the group. When the number of adult males is greater than the number of females, this will increase the competition between adult males within the group [21]. In the infant stages, the survivability of the infant is influenced by the mother's infant rank. The sons of high-ranking mothers had better survivability than the sons of the low-ranking mother.

Moreover, high-ranking mothers give a higher male infant proportion than low-ranking mothers. For female infants or daughters, mother rank did not significantly affect their survivability. Also, there is no sex difference between sons and daughters in their survivability. Furthermore, the interval of interbirth of daughters is longer than sons. Based on the Triver-Willard hypotheses, interbirth intervals suggest that the deviation in birth sex ratio is already established at conception [22]. These previous studies showed that the sex ratio is influenced by the proportion between the ranking of mothers and interbirth intervals that happened when each individual in the group was born.

Natality and Mortality

Table 3 presents the natality rate of each population of LTMs found in Ir. H. Djuanda GFP. The natality rate is represented by the *crude birth ratio,* i.e., the ratio between the number of infants and productive females in a group. The crude birth ratio of the LTMs population is in the range of 0.50 – 0.60, with a mean of 0.55. This result is similar to the studies conducted in Paliyan Wildlife Reserve and Kaliurang Nature Tourism Park, Yogyakarta, which found a natality rate of 0.40 – 0.60 [23].

Each adult female individual can give birth to 1 baby and rarely two babies every year, with pregnancy occurring every 1.5 – 2 years [23]. In addition, the high birth rate is also influenced by the role of productive females during treatment. Productive females are divided into experienced and inexperienced females. A higher proportion of experienced females results in a better survival rate for infants born [24]. On the other hand, LTMs have been reported as animals that associate food availability and birth rate. As long the area they occupy has an adequate abundance of feed, the birth rate will remain stable [25].

The survival rate approaches the mortality rate of the LTMs population. The survival rate is the ratio between the number of individuals from one age class who survive and move to the next age class. The mortality rate for all LTMs groups is listed in Table 4. The highest mortality rate is in the sub-adult age class (M_{SA-A}), with an average of 0.71. This condition was also found in a study conducted in Lampung Province, where the mortality rate for the young adult class ranged from 0.69 to 0.76. This value can be linked to the competition in the group when maintaining social status. Fights between individuals potentially decrease the number of individuals in the adult age class [18]. In addition, the survival rate is also influenced by ecological constraints that affect the behavior of each ranking. For example, we found that predation risk decreases with increased canopy height. When a group occupies some tree, high ranking-mother will dominate the higher position on the canopy tree and food proportion more than a low-ranking mother [26].

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Table 1. The LTMs Group Composition

NO	I	J		SA		A		Tot
		M	F	M	F	M	F	
1	4	3	3	3	5	4	3	**25**
2	5	4	4	6	5	3	4	**31**
3	7	6	5	6	7	5	5	**41**
4	6	5	4	4	7	5	5	**36**
5	7	5	6	5	8	5	5	**41**
6	9	7	7	9	9	8	6	**55**
Tot	**38**	**30**	**29**	**33**	**41**	**30**	**28**	**229**

M: Male; F: Female; I: Infant; J: Juveniles; SA: Sub-adult; A: Adult

Table 2. The LTMs Sex Ratio

No	Male	Female	Sex Ratio
1	10	11	1 : 1.1
2	13	13	1 : 1
3	17	17	1 : 1
4	14	16	1 : 1.1
5	15	19	1 : 1.3
6	24	22	1.1 : 1
Total	**93**	**98**	**1 : 1.1**

Table 3. Crude Birth Ratio of LTMs

No	Infant	Productive Females	Crude Birth Ratio
1	4	8	0.50
2	5	9	0.56
3	7	12	0.58
4	6	12	0.50
5	7	13	0.54
6	9	15	0.60
Total	**38**	**69**	**0.55**

Table 4. Mortality Rate of LTMs

Point	Mortality		
	M_{I-J}	M_{J-SA}	M_{SA-A}
1	0.14	0.17	0,75
2	0.04	0.38	0,67
3	0.14	0.30	0,70
4	0.25	0.13	0,70
5	0.08	0.33	0,74
6	0.02	0.36	0,72
Average	0.11	0.28	0.71

M_{I-J}: Mortality rate from Infants to Juvenile
M_{J-SA}: Mortality rate from Juveniles to Sub-adults
M_{SA-A}: Mortality rate from Sub-adults to Adults

DOI: 10.5614/3bio.2022.4.1.2

Age-class ratio

The population structure of LTMs in the study area based on age class is presented in Figure 3. Figure 3 shows that each group at the observation point has quite a similar age class ratio. The population structure of LTMs in Ir. H. Djuanda GFP shows a *regressive pattern*, with an average proportion of infants 16.59%, juveniles 25.76%, sub-adults 32.31%, and adults 25.33%. This pattern is characterized by fewer individuals in the younger group and tends to increase in the older age class [25]. A declining population generally indicates an unstable growth, which will decrease the population and become extinct. However, this can also be caused by the qualitative method of grouping age classes. It is often difficult to estimate the age of animals in nature. The classification usually uses morphological characteristics. Hence, the age class does not have the same age range and causes an accumulation of individuals in a certain age class [20].

LTMs's Population Growth

Based on the Lefkovitch matrix modeling results, the population growth for each LTMs group is listed in Figure 4. The estimate of the total population of LTMs in Ir. H. Djuanda GFP in 2046 is 1128 individuals, or about 4.92 times the population in 2021. The annual population growth rate for each group is 15-25%, with an average of 19.7%. This growth rate is comparable to one of the LTMs population in Paliyan SM and Kaliurang Forest, i.e., 20-30% [20], and much higher than Peucang Island, i.e., 11.51% [12]. The growth rate difference can be related to many factors, particularly to the variation in population structure and habitat characteristics. Population structure can be in age class proportions, and the proportion of female parent ranks in the group as described in the previous section. Habitat characteristics determine the abundance of forage sources, which affect the population size and birth rate of a group. For example, there has been an increase in the group size of LTMs in areas that are increasingly disturbed by human activities, such as a study conducted in Lampung province [17].

3.2. Minimum Viable Population Size

The MVP of each group of LTMs is presented in Figure 5. The average MVP of each class is 11 for infants, 7 for juveniles, 11 for sub-adults, and 9 for adults. The MVP size of each group is generally almost the same as the current year group size, with an average of 38 individuals per group. When calculated in one area, the MVP of LTMs is 230 individuals with an average group size of 38 individuals.

3.3. Preparation of Population Control Scheme

Based on Indonesian Government Law No. 28 2011, the Grand Forest Park area can use wild animals for research and scientific activities or population development, such as in captivity. In addition, the use of wild animals for trading purposes can be carried out on wild animals that are not protected, either from captivity or capture from nature. Some of the animal utilization activities mentioned above may be carried out while maintaining the preservation of the population of related animals in their natural habitat.

LTMs are not classified as protected animals in Indonesia; thus, controlling the LTMs population using several treatments, such as relocation and captive activities for trading, are allowed. Especially for captive activities, it can only be carried out after the issuance of a captive and trading activity permit by providing information on population demographics, habitat conditions, and harvest quotas (The Regulation on Forestry Minister no 19 2005). For LTMs trading activities, these animals must come from captivity that has special treatment, from maintenance to post-harvest inspection, according to the CITES Annex A30 file about *Macaca fascicularis*.

According to Ir. H. Djuanda GFP manager, LTMs problems are caused by the low carrying capacity of the area, the absence of natural predators, and the existence of tourism, residential and agricultural activities both inside and outside the area. This information is obtained from monitoring around the area and reporting by local communities, which the management accommodates. Currently, there is no specific program for controlling the LTMs population. There was a screening activity of 200 monkeys in 2018 through the assistance of the Provincial Conservation Agency. However, the population control activities did not use certain references, such as the use of MVP. Regarding the LTMs problem, the management is open to any recommendations regarding population control activities that can be carried out in the Ir. H. Djuanda GFP.

Based on the above regulation, this study suggests two schemes of LTMs population control: age class control and colony control. Age class control is applied for the age class whose size has passed the MVP size, while colony control is applied when the size of all age classes in a group/colony has passed the MVP size. Furthermore, the implementation of population control activity cannot be done next year. Although the LTMs population in 2021 is almost the same as the MVP in general, there are differences in the age class ratio between the two population demographics. The MVP has a larger proportion of infant age classes than older age classes, while the 2021 LTMs population age class ratio has the opposite condition. Population control can only be carried out after several years after all age classes have passed their MVP.

DOI: 10.5614/3bio.2022.4.1.2

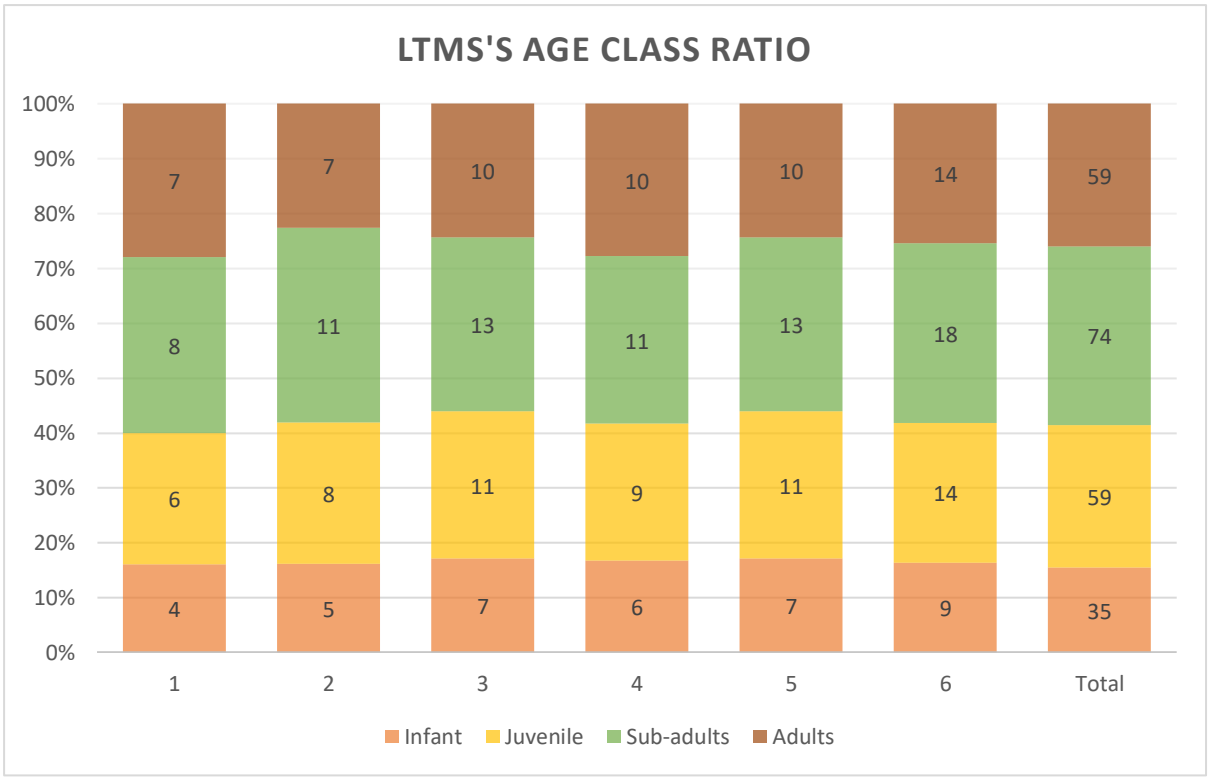

Figure 3. Age-class Ratio of LTMs

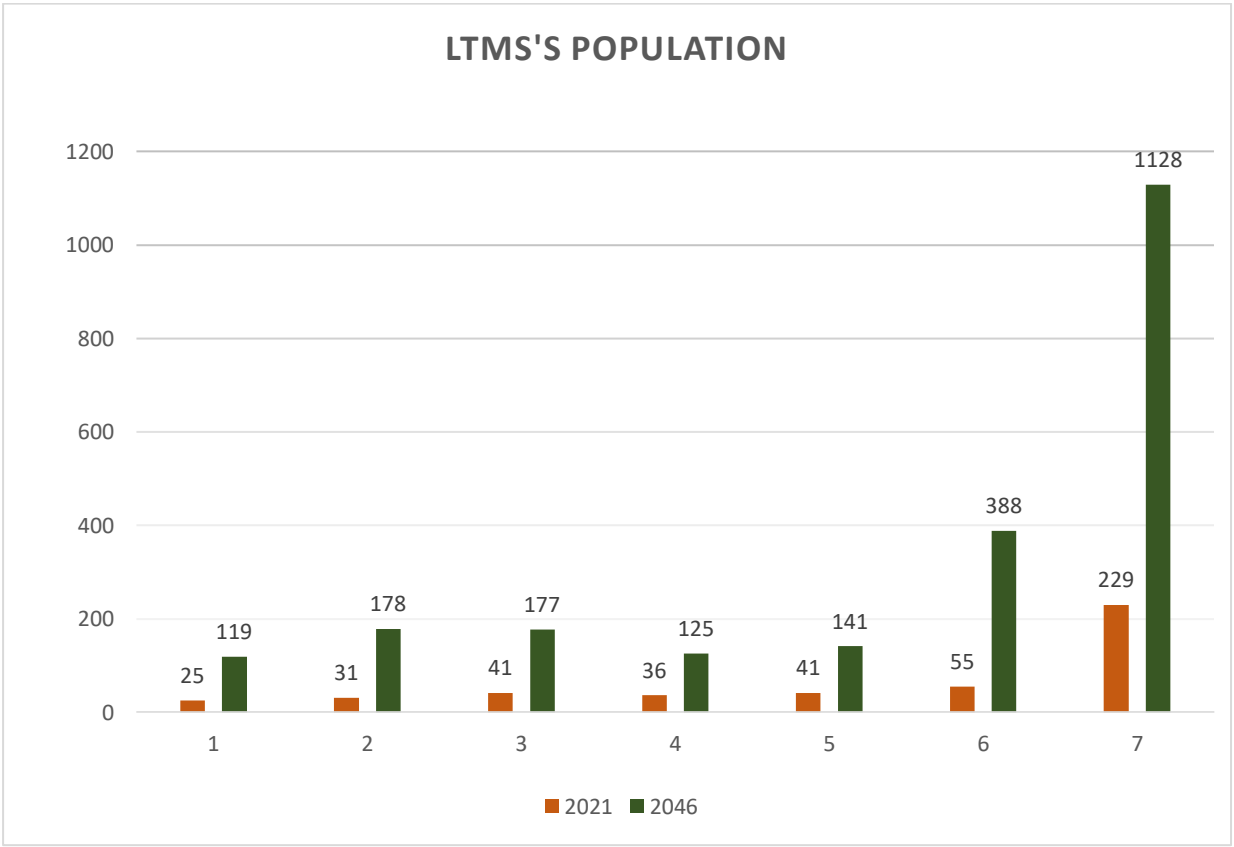

Figure 4. Comparison of LTMs Population Size in 2021 and 2046 based on current growth rate

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Based on the simulation of population growth and MVP application both in age class and in groups, the details of the time, the number of individuals, and control costs of each scheme are shown in Table 5. In the age class control scheme, the total controlled population for 25 years is 334 individuals, with an estimated expenditure of IDR 66,800,000.00 and an estimated gross income from captive activities of IDR 567,800,000.00-. In the colony control scheme, the total number of controlled population for 25 years is 406 individuals, with an estimated expenditure of IDR 81,200,000.00 and an estimated gross income from captive activities of IDR 690,200,000.00-. However, there is no certainty of the success of this activity given the availability of inadequate data. Therefore, population control measures need to be carried out with other actions, such as providing new feed areas and limiting zones with human areas, making no-feeding and no-littering boards. This is intended to minimize as much as possible the interactions that can occur between LTMs and humans. When interactions between humans and LTMs can be limited, access to anthropogenic feed is cut off, and LTMs will return to consuming natural feed available in the area [1].

4. Conclusion

The MVP of LTMs in Ir. H. Djuanda GFP is 230 individuals, with an average group size of 38 individuals. The average MVP for each age class is 11 for infants, 7 for juveniles, 11 for sub-adult, and 9 for adults. Population control schemes can be carried out by age class control and colony control. The total number of controlled individuals for the next 25 years in the age class control scheme is 334 individuals, while in the colony control scheme is 406 individuals. This study recommends integrated management of wildlife populations that combines population control and income generation from legal animal trading.

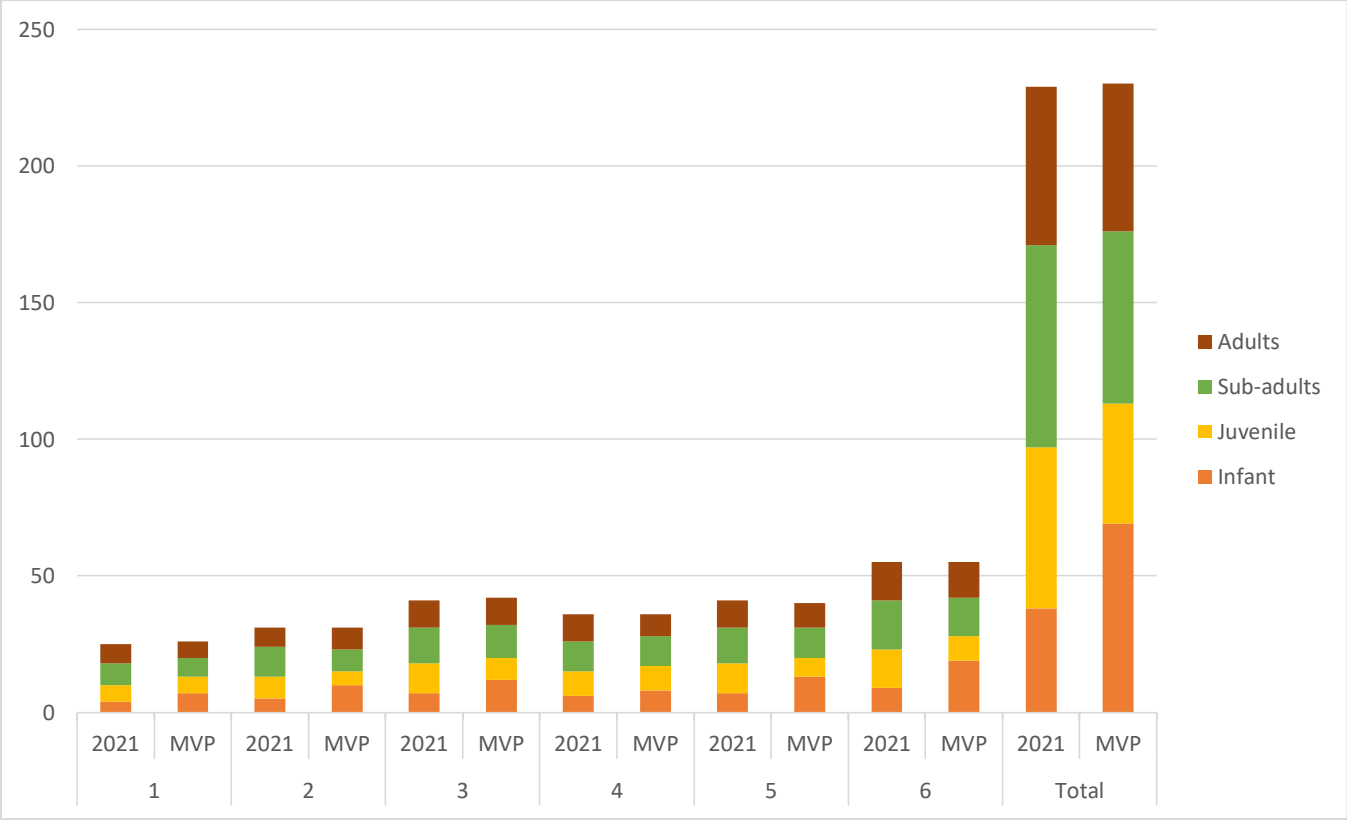

Figure 5. Comparison of LTMs Population Structure in the Year 2021 and MVP

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Table 5. Comparison between LTMs Population Control Scheme

No	Years Control		Cost estimation (IDR 200.000 @ Ind., during 25 Yrs)			
	Age-class Control	Colony Control	Age-class Control		Colony Control	
			Ind.	Cost	Ind.	Cost
1	J: 9,10,13,15,18,20,22,25 SA :0 A: 0,1,2,4 – 25	every 7 years, starting from the 9th year	35	IDR 7,000,000.00	43	IDR 8,600,000.00
2	J: every years SA: 0 A: every odd year from 1st years	every 7 years, starting from the 8th year	58	IDR 11,600,000.00	67	IDR 13,400,000.00
3	J: 0,2-8, 10, 12-17, 21-25 SA:0 A: every years	every 10 years, starting from the 11th year	50	IDR 10,000,000.00	60	IDR 12,000,000.00
4	A : every years	every 12 years, starting from the 12th year	29	IDR 5,800,000.00	52	IDR 10,400,000.00
5	J: every years SA:0 A : every odd year	every 9 years, starting from the 10th year	45	IDR 9,000,000.00	48	IDR 9,600,000.00
6	J:every years SA: 0 A: 0-2; 4,6, etc., with every even year	every 4 years, starting from the 6th year	117	IDR 23,400,000.00	136	IDR 27,200,000.00
Total			**334**	**IDR 66,800,000.00**	**406**	**IDR 81,200,000.00**
Estimated Gross Income From Captive Activities			**IDR 567,800,000.00**		**IDR 690,200,000.00**	

I: Infant; J: Juveniles; SA: Sub-adult; A: Adult

Acknowledgements

The authors thank the UPTD Ir. H. Djuanda GFP as the area manager for the great support of this research.

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DOI: 10.5614/3bio.2022.4.1.2

e-ISSN: 2655-8777 http://journals.itb.ac.id/index.php/3bio

Autosterilization Effect of Gamma Radiation in Non-sterile Radiopharmaceuticals

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Received: 2021-10-12
Accepted for publication: 2022-03-01

Abstract

Gamma radiation is commonly used in sterilizing various products because of its microbial-killing property. Several radiopharmaceuticals are processed aseptically, which requires a well-managed GMP-based facility. This study aims to ensure that radiopharmaceutical can behave as an auto sterilizing agent since it contains radionuclide that emits gamma radiation. Sm-153-ethylenediaminetetramethylenephosphonate (Sm-153-EDTMP) and I-131-Hippuran were used as models in this study, in which various radioactivities of these products were added into non-sterile vials in the non-aseptic working area and tested for sterility using direct inoculation method. The result showed that samples containing 7 mCi of Sm-153-EDTMP and less than 2 mCi of I-131-orthoiodohippurate (I-131-Hippuran) changed the clarity of the media, but not for those containing higher radioactivity. The results showed that Sm-153-EDTMP and I-131-Hippuran at certain radioactivity can auto sterilize themselves, whereas the positive control sample and the products diluted with non-sterile water remained unsterile. This study showed that radiopharmaceuticals have auto sterilizing properties at relatively low radioactivity, depending on the products' bioburden. Therefore, the usual aseptic preparation of some radiopharmaceuticals can be considered terminal sterilization.

Keywords: Sm-153-EDTMP, I-131-Hippuran, gamma radiation, autosterilization, radiopharmaceutical, radioactivity

1. Introduction

Radioactive sterilization, e.g., gamma rays, electron beams, ultraviolet light, and X-rays, is commonly used in pharmaceutical industries on sterilizing active ingredients in drugs. This method is chosen since the physicochemical properties of active ingredients in drugs remain. Among all methods, gamma irradiation using a radioisotope source (e.g., cobalt-60 and cesium-137) is preferred since it penetrates lower in the object materials. So far, gamma-ray irradiation with a dose of 25 kGy is more commonly used in which one kGy is equivalent to one joule/gram of matter. The sterilization using radiation can damage or induce the rupture of microbial cells that will eventually kill the cell. Besides damaging DNA as the main target, the cell membrane can also be affected. The water irradiation will trigger the formation of water-derived radiolysis products, including H_2O, H_2O_2, and $^{\cdot}H_2$. In turn, strong oxidants formed, e.g., peroxide and free radical species, can damage microbial cells' DNA and lead to cell death [1-6].

Most radiopharmaceuticals used in nuclear medicine studies are short half-lives, allowing their release before sterility testing is completed. Furthermore, a short radionuclide half-life (e.g., under 20 minutes) allows radiopharmaceutical preparation on the patient to be administered online through a validated production system. If the radiopharmaceutical uses a validated aseptic process, the omission of radioactive drug products needs to be justified before completing the sterility test [7].

Because of their specific formulation, radiopharmaceutical kits cannot be terminally sterilized by heat or other methods and should be processed aseptically. Its manufacturing process requires compliance with cGMP regulation, i.e., conducted in a particular facility being aseptic within a Class A room inside Class B room. Operators who carry out the process must wear specific gowning. All equipment involved, such as containers, packaging material, equipment, and clean room, must be sterilized. Obtaining an aseptic manufacturing process that conforms to the cGMP facility is not easy and requires

DOI: 10.5614/3bio.2022.4.1.3

commitment, high budget, planning, and maintenance [8-9].

Gamma-emitting radionuclide Technetium-99m or Tc-99m is known to have the sterilizing capability on kits that are prepared to be labeled. A study to prove this characteristic was previously reported in which tetrofosmin kits were used as a model. Other studies also reported that the sterilization effect was dose-dependent [6, 10]. In an experiment using tetrofosmin kits, 10 mCi of Tc-99m was a minimum dose showing the sterilizing effect [10]. A similar study using other radiopharmaceuticals or radionuclides should be conducted to support this conclusion. Sterility tests according to pharmacopeia can be conducted through direct inoculation (immersion) and membrane filtration. The sample needs to be added aseptically in bacterial and fungi culture media to be incubated minimum 14 days at 30-35^{o}C and 20-25^{o}C, respectively [11-14].

FDA has already approved a rapid and automatic instrument for sterility testing that uses a direct inoculation method or immersion. The instrument works on the basis of biochemical or physiological growth parameters measured in a liquid medium. For instance, CO_2 production can be detected using colorimetric methods or measurement of headspace pressure changes. Another instrument also developed to test the sterility system using direct fluorescent labeling techniques and solid-phase laser scanning cytometry to count living microorganisms. Other validated innovations in sterility testing systems are also widely available [15-20].

This study aims to substantiate the argument that radiopharmaceuticals or radiolabeled compounds can behave as auto sterilizing agents at certain radioactivity. Samarium-153 (Sm-153) and iodium-131 (I-131) labeled radiopharmaceuticals in the form of Sm-153 ethylenediaminetetramethylenephosphonate (Sm-153-EDTMP) and I-131 orthoiodohippurate (I-131-Hippuran) were used as models. Although the best sterilizing method for pharmaceutical products is terminal sterilization, not all products can be treated this way, and some need to be processed aseptically. On the other hand, the aseptic process requires a lot of efforts, such as a well-designed facility and high-cost maintenance. By ensuring that gamma emitted radionuclide can act as an auto sterilizing agent, radiopharmaceutical preparation with filtering through a microbial filter instead of autoclaving is sufficient to produce a sterile product.

2. Material and Method

2.1. Materials

Materials used in this study were, i.e., Fluid thioglycolate (FTG, Difco), Tryptic Soy Broth (TSB, Bacto), Tryptic Soy Agar (TSA, Difco), cultures of *Staphylococcus aureus* and *Aspergillus niger*, radiopharmaceutical products (Sm-153-EDTMP and I-131-Hippuran (PTRR-Batan)), 0.22 µm Millipore filter (Millex-GS®), water for injection (Ikapharmindo), water purification system equipment (Merit), dose calibrator (Atomlab-300), glove box (Comecer), fume hood equipped with lead glass, disposable syringes, pipettes, and glasswares.

2.2. Methods

2.2.1.Preparation

We used fluid thioglycolate as bacterial growth medium, Tryptic Soy Broth as fungal medium growth, and Tryptic Soy Agar to monitor a sterile environment. The microorganisms studied were *Staphylococcus aureus* and *Aspergillus niger*. We used demineralized water (demin water) obtained by purifying the tap water through a water purification system.

Sm-153-EDTMP of various radioactivity was added into a series of clean but unsterilized vials without filtration, and a dose calibrator measured the radioactivity. The same treatment was applied to a sample of I-131-orthoiodohippurate (I-131-Hippuran). This preparation of samples was carried out inside a fume-hood equipped with lead glass to protect the operator from radiation exposure.

The same amount of Sm-153-EDTMP and I-131-Hippuran was added into a sterile vial through a 0.22 µm filter. The preparation was carried out inside an isolator (glove box) previously cleaned, sanitized, and stored for several days to decrease the radioactivity. Each filtered Sm-153-EDTMP and I-131-Hippuran were used as negative controls. Filtration of the samples through 0.22 µm porosity filter (Millex-GS®) is the main step in the aseptic process, which means sterilizing the products. A vial containing 2 mL of tap water was used as a positive control.

Various radioactivity of Sm-153-EDTMP, i.e., 7 mCi, 15 mCi, and 30 mCi was added into 3 vials that were previously prepared clean but not sterile. Afterward, the vials were stored in a fume-hood for ~ 2 weeks to decay the radioactivity. Various radioactivity of I-131-Hippuran i.e., 0.5 mCi, 1 mCi, 2 mCi, 5 mCi and 8 mCi was pipetted into 5 cleaned but non-sterile vials. The vials were then put into a lead-shield container and allowed for ~ 2 weeks in a fume-hood. The same radioactivities of Sm-153-EDTMP were also added into 3 vials, then added with 2 mL of non-sterile water, followed by the same treatment as the latter mentioned above.

In an Erlenmeyer flask, 6.0 g of FTG was added with 200 ml of demin water. The mixture was heated to dissolve. 15 ml of the mixture was put into test tubes covered with a cotton cap. The tubes were then sterilized using an autoclave at 121°C for 20 minutes. The same amount of TSB was prepared in a similar manner. Approximately 8.0 g of TSA

DOI: 10.5614/3bio.2022.4.1.3

was prepared in an Erlenmeyer flask with 200 ml of demin water. The mixture was heated and dissolved, sterilized using an autoclave at 121°C for 20 min, and 20 ml of it was placed aseptically into a sterile petri dish. Before the solution is utilized, these media must be tested for growth promotion to determine their suitability in sterility testing and ensure their ability to support microbial growth. *S. aureus* was added to test tubes with FTG and TSA. Meanwhile, *A. niger* was only added to test tubes with TSB. Afterward, all tubes and plates were incubated at a suitable temperature for 5 days. The validity of media obtained when turbidity occurred or colonies were formed; thus allowing the media to be used. Tubes containing FTG and a half of TSA plates were stored at 30-35°C and TSB tubes and a half TSA plates were stored at 20-25°C for 5 days before use [3,8,10,11].

Disinfection of the glove box used in sterility testing was done by cleaning it with savlon and 70% alcohol and left for 3 hours prior to use.

We carried out a sterility test using the direct inoculation method. Several tubes were added with samples and controls and FTG and TSB, respectively [11-14]. The sterility test was carried out in an isolator (glove box) which had been previously cleaned and sanitized, and during the test session sterile environment was monitored using TSA agar plates afterward; the environmental monitoring plates were incubated for 20-25°C up to 3 days, observed for microorganisms' growth within the days, alternatively in 30-35°C for 2 days [9].

We aseptically transferred 1 mL of each sample into tubes containing TSB and FTG, respectively. The TSB tubes were incubated at 20-25°C and FTG tubes at 30-35°C for 2 weeks. The turbidity or sample clarity was observed daily. A similar procedure was also done to a vial of sterile Sm-153-EDTMP and I-131-Hippuran as negative controls and a vial containing 1 mL of tap water as a positive control. The sterility test was done in a TSB tube with fungi *A. niger*. A tube containing FTG with bacteria *S. aureus* was also prepared and acted as a reference.

The growth of microorganisms was marked by turbidity in the media tubes, which indicated a positive result. The sterile media remained clear, indicating a negative result. The experiment was done in three replications.

3. Results and discussion

This study used a validated culture media through growth promotion testing (fertility testing) and turbidity test (Table 1). In this validation, culture media that have been added with 10-100 CFU of bacteria and fungi must produce colonies of bacteria and fungi within 5 days [14].

The autoclave-sterilized culture media was stored in a temperature-adjusted incubator, i.e., 20-25°C for TSB and TSA, and 30-35°C for FTG and TSA for 6 days. Turbidity was neither demonstrated in the fluid media nor in TSA plates (Table 2). Therefore, the media were approved and can be used for sterility testing.

Table 1. Growth promotion testing for culture media

Microorganism	**Aerobe bacteria (*Staphylococcus aureus*)**	**Fungi (*Aspergillus niger*)**
Temperature of incubation	30-35°C	20-25°C
Incubation period	5 days	5 days
Result (growth)	positive	positive

Table 2. Sterility observation of culture media before use

Culture media	**Sign of bacterial growth at**	**Sign of fungal growth at 20-25°C**
FTG tubes	negative	N/A
TSB tubes	N/A	negative
TSA plates	negative	negative

Furthermore, we have found that within 14 days of observation, the media's clarity did not change despite the addition of sterile samples to both the TSB and FTG media. By contrast, the non-sterile sample changes the two media from clear to turbid within a day of observation. This finding suggests that both samples can be used as negative and positive control, respectively.

Samples of I-131-Hippuran in non-sterile vials and inoculated into TSB and FTG showed no turbidity, similar to the negative control sample. In contrast, those with radioactivity of 0.5 and 1.0 mCi showed turbidity in both media, similar to the vial sample containing water as positive control (Table 3, Figures 1 and 2). These results indicate that radioactivity of I-131 from 2 mCi can sterilize itself. It is likely that I-131 which has higher energy than Tc-99m can act as an auto-sterilizing agent in much lower radioactivity [10].

Three replications of Sm-153-EDTMP samples with various radioactivity showed a significant difference. Those added with 7 mCi caused turbidity, while those with 15 mCi and 30 mCi remained clear and served as negative control (Figure 3 and 4, Table 4). On the other hand, the same series

DOI: 10.5614/3bio.2022.4.1.3

of samples diluted with non-sterile water showed turbidity (Table 4). Thus, the radioactivity of Sm-153 from 15 mCi can sterilize Sm-153-EDTMP, while radioactivity of 7 mCi cannot, and all samples with various radioactivity added with non-sterile water showed turbidity. The results showed that the level of radioactivity can auto sterilize to such an extent as long as the container is clean. However, the work is carried out in a fume-hood or regular work area. So the auto sterilization effect of radiopharmaceuticals depends on the level of radioactivity, and the cleanliness level of the medium since all the samples diluted with non-sterile water showed turbidity, as can be seen in Table 4.

Table 3. Sterility test on I-131-Hippuran with various radioactivity packed in unsterilized vial

Sample	**Bacterial growth (30-35°C, 14 d)**	**Fungal growth (20-25°C, 14 d)**
Sterile I-131-Hippuran (negative control)	-	-
Non-sterile water (positive control)	+	+
0.5 mCi of I-131-Hippuran	+	+
1 mCi of I-131-Hippuran	+	+
2 mCi of I-131-Hippuran	-	-
5 mCi of I-131-Hippuran	-	-
8 mCi of I-131-Hippuran	-	-

Note : - : clear, indicated no microbial growth
+ : turbid, indicated microbial growth

Table 4. Sterility test on Sm-153-EDTMP with various radioactivity packed in unsterilized vial, with and without dilution

Samples	**Diluted samples**		**Undiluted samples**	
	Bacterial growth (30-35°C, 14 d)	**Fungal growth (20-25°C, 14 d)**	**Bacterial growth (30-35°C, 14 d)**	**Fungal growth (20-25°C, 14 d)**
Sterile Sm-153-EDTMP (negative control)	-	-	-	-
Non-sterile water (positive control)	+	+	+	+
7 mCi of Sm-153-EDTMP	+	+	+	+
15 mCi of Sm-153-EDTMP	+	+	-	-
30 mCi of Sm-153-EDTMP	+	+	-	-

Note : - : clear, indicated no microbial growth
+ : turbid, indicated microbial growth occurred

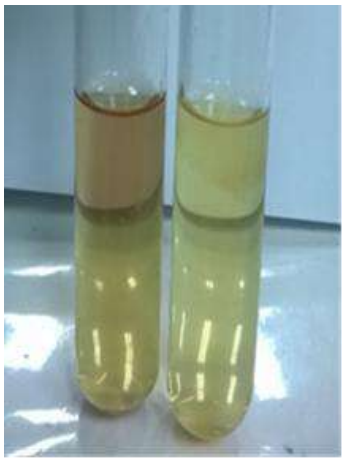


Figure 1. Performance of negative control (left) and positive control (right) in the I-131-Hippuran experiment in FTG media

DOI: 10.5614/3bio.2022.4.1.3

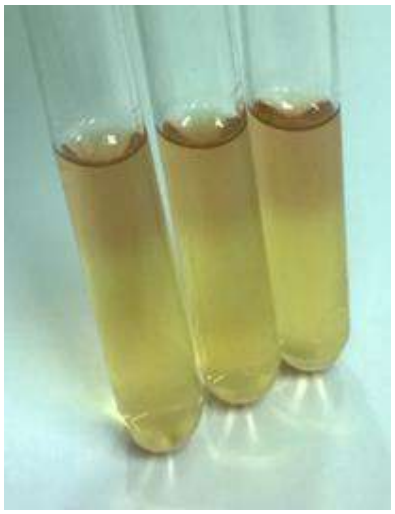
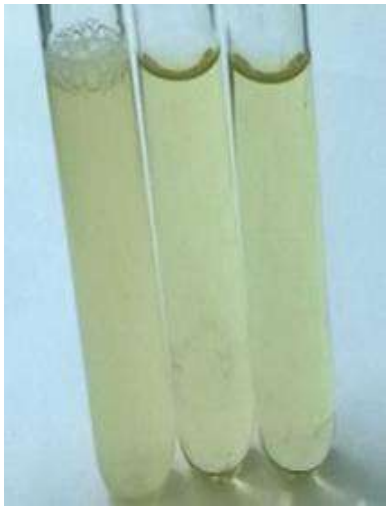


Figure 2. Performance of I-131-Hippuran samples of 2 mCi (left), 5 mCi (middle) and 8 mCi (right) in which all of them showed clear in FTG media

Figure 3. Performance of negative control (right) and positive control (left) in the Sm-153-EDTMP experiment in TSB media



Note : The turbidity which denotes insterility can not be shown clearly from these figures but it is always accompanied by foam at the surface of the media as shown clearly in these pictures above

Figure 4. Performance of Sm-153-EDTMP samples of 7 mCi, 15 mCi and 30 mCi, from which only one of them showed turbid (left) in TSB media

DOI: 10.5614/3bio.2022.4.1.3

Bioburden likely contributes to the effectiveness of this radiation auto sterilization process. The term bioburden informs the number and types of viable microorganisms present inside a product before sterilization. The radiation sterilization process will be more effective when the bioburden is low. As a requirement for common parenteral pharmaceutical products, the sterilization process and aseptic technique are to produce sterile products that conform to the sterility assurance level (SAL), which is usually smaller than one in one million units tested (10-6). SAL is derived from kinetic studies on the probability of living microorganisms on or inside a product after sterilization, known as microbial inactivation [4].

The mechanism of action can be direct or indirect. In the direct radiation effect, the ionizing radiation damages the DNA strands of microbial cells. In contrast, in the indirect radiation effect, the radiation interacts with water molecules, producing free radicals and peroxy radicals. These radicals can damage DNA and hamper cell reproduction, leading to microorganisms' death. Indeed, a liquid solution increases the microorganisms' sensitivity compared to a frozen state. In a frozen state, free radicals are immobile, preventing them from diffusing, thus preventing indirect radiation effects. Free radicals can be reactive to oxygen molecules, leading to the production of peroxy radicals that can damage biological cells. Thus, free water radicals yield is much lower in low water activity or dry conditions and microorganisms are becoming more resistant. Still, ionizing radiation becomes greater in wet material since gamma radiation's mechanism generates free hydroxyl radicals and is radiotoxic. Hydroxyl radicals are well-known as strong oxidants that can damage DNA strands and the chemical bond of molecules, either in microorganism cells or in other materials [4,5].

Both radionuclides, i.e., samarium-153 and iodium-131 which were used as models, have proven that radiopharmaceuticals labeled with gamma-emitting radionuclides can behave as an auto-sterilizing agent. One of the weaknesses of this method is the difficulty in identifying microorganisms at the beginning of the killing process. As per safety considerations, the sterility test can only be done after the radioactivity has declined.

Gamma irradiation technology has been widely used to sterilize food and medical devices effectively. However, a recent publication reported the side effect of using gamma radiation to sterilize allografts that, to some extent, can alter the physicochemical properties of sterilized material. Another publication also reported several animal studies that demonstrated that irradiated food consumption provoked genome instability and more likely mutagenic effects that could potentially induce cancer. These findings of the oncogenic potential of irradiated consumables strongly suggest that new, long-term, prospective clinical studies should be conducted soon to investigate whether irradiated food is safe for human consumption [4,5]. By understanding this phenomenon, it can be justified that radiopharmaceuticals commonly used in hospitals exhibit self-protection against microorganisms, so the aseptic manufacturing process of this product is not necessarily applied strictly because its auto sterilization property can be considered terminal sterilization.

4. Conclusion

Samarium-153 and Iodium-131, as gamma-emitting radionuclides labeled to pharmaceutical products at certain radioactivity, can sterilize themselves and act as an auto sterilization agent. Final filtration of radiopharmaceuticals in the manufacturing process inside a clean isolator and using sterilized glasswares and vials for packaging is sufficient to deliver sterile products. It is unnecessary to manufacture radiopharmaceuticals in a clean room with Class A classification, since the characteristic of gamma emitting radionuclide intact in the radiopharmaceutical can sterilize the product. The process can be considered a terminal sterilization process. This is a justification to loosen the existing regulation of the aseptic manufacturing process for radiopharmaceutical products in comparison with the one for aseptic non-radiopharmaceutical parenteral products.

Acknowledgements

Many thanks to the Head of PTRR-BATAN and Head of Radiopharmaceutical Technology Division for supporting this work. High appreciation to all colleagues who participated in this project.

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DOI: 10.5614/3bio.2022.4.1.3

e-ISSN: 2655-8777 http://journals.itb.ac.id/index.php/3bio

Wheatgrass microgreen with high antioxidants content in an urban indoor farming system

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Received: 2021-08-31
Accepted for publication: 2022-01-02

Abstract

Urban lifestyle is identical to stressful life and sedentary habit, leading to the increase of chronic conditions such as diabetes and cardiovascular-related diseases. Antioxidants are renowned for maintaining cellular function by quenching radicals produced in stressful conditions or infection. Fresh fruits and vegetables are the primary sources of antioxidants, but the long postharvest and transport system may reduce the benefits for the urban population. Hence, we designed a cultivation method to produce wheatgrass microgreens with high antioxidants in an urban indoor farming system. Generally, plants require light at the wavelength of 663 and 642 nm (red) and 430 nm and 453 nm (blue) to allow photosynthesis and production of secondary metabolites, such as antioxidants. We applied the LED lights with an RGB ratio of 91R/9B, 83R/17B, 47R/53B, 35R/65B, and white florescent as the control. Our results showed that 91R/9B reduced fresh mass and chlorophyll content, which might be due to the suppression of photosynthesis capacity. Interestingly, we found a significant ($p<0.05$) increase in carotenoids and flavonoid contents due to light combinations of 35R/65B and 83R/17B, respectively. However, the total antioxidants capacity was similar among all treatments. Carotenoids and flavonoids are among the antioxidants with a significant role in decreasing the risks of chronic diseases and their potential as antiviral agents. This cultivation system of wheat microgreen could be a promising solution to routinely supply carotenoids and flavonoids to the urban population. Further, it is also considered more environmentally friendly as it could be performed in a limited amount of land (vertically) and potentially use less energy for distribution.

Keywords: blue light, carotenoids, chlorophyll, flavonoid, red light

1. Introduction

Sedentary lifestyle, food habits, and stressful living conditions were reported to cause chronic oxidative stress, resulting in chronic lifestyle diseases [1], the top causes of death for the global urban population. The poor intake of nutritious food coupled with constant exposure to environmental pollution or prolonged stressful condition with minimum exercise leads to oxidative stress [2]. Oxidative stress is a phenomenon caused by an accumulation of free radicals, which are by-products of metabolic processes in the biological systems. The excess presence of reactive oxygen species (ROS) in cells causes negative effects on proteins, lipids, and nucleic acids. Cells in the human body can naturally activate antioxidant defense systems by producing enzymes such as superoxide dismutase (SOD) and catalase (CAT) to prevent ROS cell damage, but only to an extent. The consumption of food rich in antioxidants may help the body in doing so [3]. Antioxidants are scavengers of free radicals in the human body, thus protecting them from chronic diseases caused by oxidative stress [4]. The consumption of food with high-antioxidant content has been linked to minimizing oxidative stress and reducing the risk of cardiovascular diseases, cancer, aging [5], neurodegenerative diseases [6], and type 2 diabetes [7]. These recent studies also emphasize that the quenching of free radicals in our body by various antioxidant compounds of fruits and vegetables is crucial and irreplicable by treating supplements containing only certain types of antioxidants.

DOI: 10.5614/3bio.2022.4.1.4

Antioxidants also hold great potential in alleviating symptoms related to viral infection. For instance, the presence of free radicals complemented by COVID-19 may increase pathogenesis in comorbid patients due to elevated oxidative stress. Therefore, COVID-19 patients are prone to decreased levels of antioxidants due to their utilization in counteracting free radicals [8]. In addition to pharmacological therapy, high-antioxidant dietary treatment is recommended in treating COVID-19 and comorbidities [9]. Hence, providing high-antioxidant food is crucial to alleviate the risks of chronic and infection diseases for either urban or rural populations.

However, the limited arable land in urban areas impedes its population's access to fresh vegetables and fruits when their antioxidant content is the highest. Furthermore, poor postharvest management and long transportation of fruits and vegetables to reach urban customers also reduce their antioxidant benefits [10]. Thus, in this research, we explored the alternative of cultivating microgreens in an urban farming system to improve antioxidant content. Microgreens are young vegetables, which could be leafy greens or fruit vegetables, that are approximately harvested at 5 – 10 cm tall [11]. They are found to contain vitamins, minerals, and antioxidants 4-6 times more than the amount found in mature vegetables. In the past few years, microgreens consumption has increased along with consumers' awareness of the importance of nutritious vegetables [12]. Microgreens can be quickly grown in a soilless medium in a short period (10-15 days), creating opportunities to be cultivated in a limited space. Therefore, microgreens have potential in urban farming because of their accessibility to urban citizens.

Wheatgrass (*Triticum aestivum*) is often grown as microgreens due to its abundance of antioxidants, promoting the consumers' health. For instance, routine consumption of wheatgrass was recorded to improve immune parameters in colorectal cancer patients [13]; protect rats from streptozotocin-induced diabetes [4]; and proposed as a potential therapeutic agent for chronic diseases [14]. Wheatgrass microgreens are typically harvested during their vegetative state (10 days after sowing). Wheatgrass grows ideally in a temperature range of 18-26 ˚C and a humidity of 40-50% [15]; [16]; [17]. In plants, photosynthesis occurs when chlorophyll pigments are present [18] and when light emits electromagnetic radiation, also known as photosynthetically active radiation (PAR), as the energy source of photosynthesis [19]. Microgreens can be cultivated using artificial light, such as light-emitting diodes (LED), which are an efficient source of light for plant cultivation due to customizable spectra, high light output, and low emission [20]. Furthermore, antioxidant content in vegetables is also regulated by the photoperiod and wavelength of the exposed lights [21]; [22]; [23]. In this research, the antioxidants content of wheatgrass microgreens was examined after a cultivation period under exposure to several light combinations. This research reveals that modulation of certain types of antioxidants by exposure to red/blue light is unique and is not always reflected by the total antioxidant capacity. The results could be the foundation for designing a system for cultivating high-antioxidant microgreens in urban households.

2. Methodology

2.1. Seed sowing and light equipment installation

Sowing was performed on an organic growth medium with a pH of 5.5 in a 35 x 10 cm tray. The bottom compartment of the tray was filled with 750 mL of water for bottom-up watering of the plants; water could reach the medium due to capillary force and then was absorbed by roots. As many as 250 grams of seeds were sowed and stored in a dark room for 3 days for germination. The trays were moved to a shelf equipped with LED installation and exposed to the lighting combinations in a photoperiod of 12 hours (h) of light and 12 h of dark for 10 days (**Figure 1F**). The LED installation consisted of a collection of red and blue LED lights, a shelf with a height of 40 cm, a power supply, and a timer. After the light installation was assembled, a black, opaque curtain was installed to block any light from going inside. The red and blue light combinations used were based on the percentage of RGB ratio, which were 91% red and 9% blue (91R/9B); 83% red and 17% blue (83R/17B); 47% red and 53% blue (47R/53B); 35% red and 65% blue (35R/65B); and white fluorescent light as the control (C). The intensity and wavelengths of each light combination or treatment are presented in **Figure 1A-E**.

Watering was made once in 3 days by adding 100 mL of water into the bottom compartment of the tray. Harvesting was done on the 10th-day post sowing (**Figure 1G**) by gently removing the whole plant from the growth medium. Plant height for each treatment was recorded 3, 6, and 10 days after sowing (DAS) by measuring from the surface of the medium until the tallest point of the plant, taken from 5 samples. Fresh biomass from 5 samples from each treatment was recorded upon harvest.

2.2. Total chlorophyll and carotenoids content

Total chlorophyll and carotenoid contents were determined using the Kirk method [24]. The extract was prepared from 50 mg of fresh wheatgrass homogenized in 10 mL acetone 80%. The extract was then centrifuged at

DOI: 10.5614/3bio.2022.4.1.4

3000rpm for 15 minutes. The obtained supernatant was disposed of, and the pellet was dissolved in 5 mL acetone 80%. The extract was centrifuged again until clear. Finally, UV-Vis measured the absorbance at 480, 645, and 663 nm. Samples were measured in duplicates, and then total chlorophyll and carotenoid contents were determined using equations 1 and 2, respectively.

$$Total\ chlorophyll\ (\mu g/mL) = 20.2(A_{645}) + 8.02(A_{663}) \quad (1)$$

$$Total\ carotenoid\ (\mu g/mL) = A_{480}) + (0.114 \times A_{663}) - (0.638 \times A_{645}) \quad (2)$$

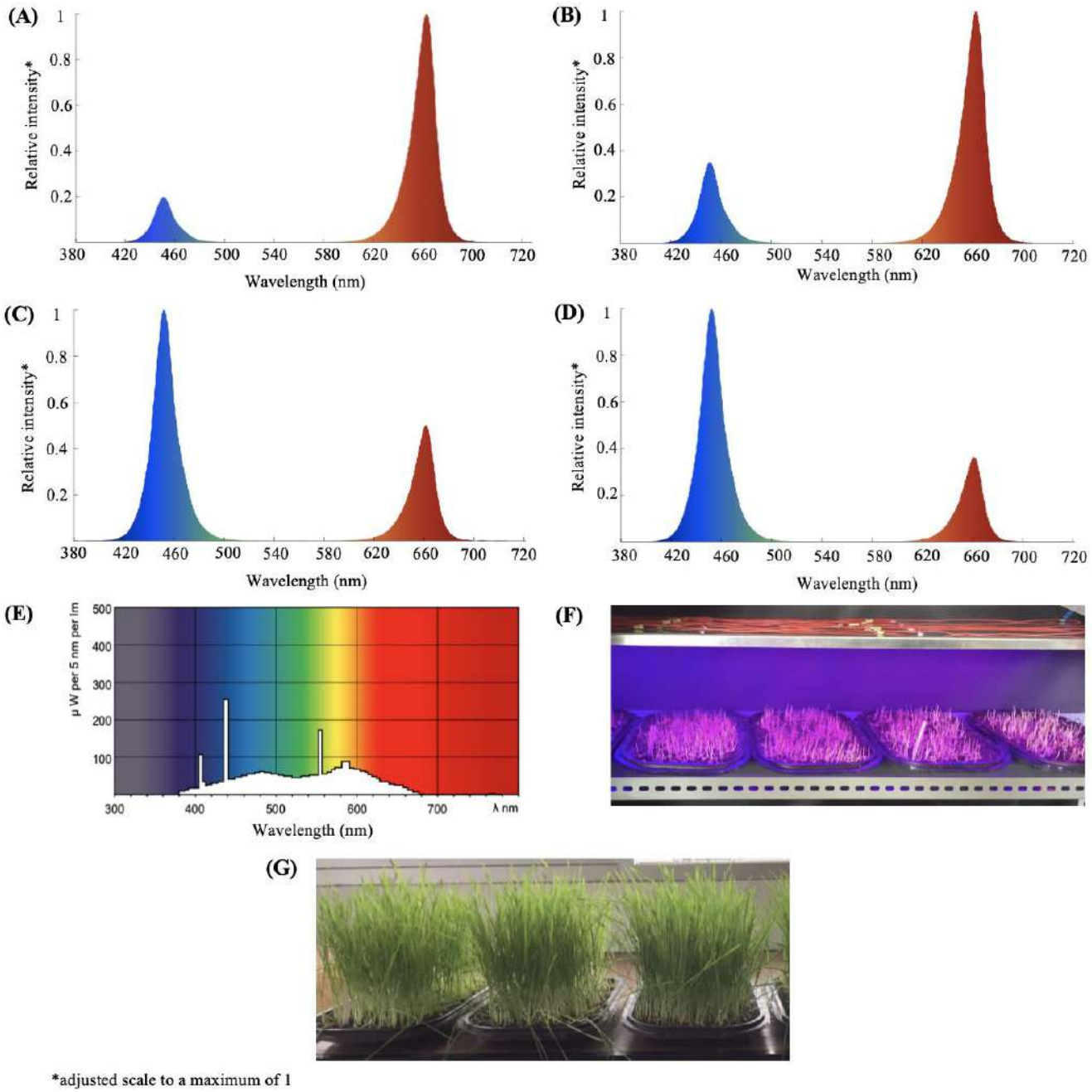

Figure 1. The intensity and wavelength of each combination of light used as the treatment in this research. The RGB color ratio of each treatment were 91% red and 9% blue (A); 83% red and 17% blue (B); 47% red and 53% blue (C); and 35% red and 65% blue (D). The trays of sown seeds were placed 40 cm under the light set up (E), and the microgreens of wheatgrass were ready to harvest on day 10 (F).

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2.3. Total flavonoid

Total flavonoid content was determined by the $AlCl_3$ assay, using quercetin as the standard solution. Around 100 mg of quercetin was dissolved in 100 mL methanol PA. 0,1 mL of standard quercetin solution (100, 200, 400, 600, 800, and 1000 µg/mL), 4 mL of distilled water, and 0.3 mL of 5% $NaNO_2$ were added to a test tube to make the standard solution. After 5 minutes, 0.3 mL of 10% AlCl3 was added. After 6 minutes, 2 mL of 1M NaOH was added. Finally, distilled water is added until the volume is made up to 10 mL, which will eventually result in a yellowish-colored solution. The absorbance is measured at 510 nm using UV-Vis, with distilled water used as the blank. Samples were performed in duplicates, and the calibration curve was plotted using quercetin as the standard. As much as 0,1 mL of wheatgrass aliquot is made by oven-drying wheatgrass at 60 ˚C for six h. The dried mass is weighed at 0.1 gram and dissolved in 10 mL aqueous methanol (1:1). The sample was then vortexed for 3 h at 150 rpm and centrifuged at 9000 rpm for 10 minutes. The obtained supernatant was stored at 4 ˚C prior to testing. The sample was then tested using the same $AlCl_3$ assay [24]. Total flavonoid content was determined using equation 3.

$$8.64E - 5x + 6.31E - 3 = 0.967 \quad (3)$$

2.4. Antioxidant activity

The DPPH assay determined antioxidant activity. Fresh wheatgrass was weighed 5 grams and then oven-dried for 72 h at 40 ˚C. Next, 0.5 grams of dry mass was extracted using 10 mL acetone 80%. The extract was vortexed at room temperature for 24 h to separate the supernatant and pellets. To make the DPPH solution, 0.002 grams of DPPH was dissolved in 25 mL acetone 80%. A 25 µL sample, 2975 µL acetone 80%, and 1000 µL DPPH solution are added into a glass vial and stored in a dark room for 30 minutes to determine the antioxidant activity. Finally, the absorbance was measured at 517 nm using UV-Vis, performed in duplicates. The blank was made by dissolving 3000 µL acetone 80% in 1000 µL DPPH solution [26]. The percentage of scavenged DPPH was determined using equation 4.

$$DPPH\ Scavenged\ (\%) = (Ab - A_{517})(Ab^{-1})(100) \quad (4)$$

2.5. Statistics analysis

The obtained data were analyzed statistically using a one-way analysis of variance and subsequently tested with the Tukey test with a 95% confidence level (p<0.05) in Python (Jupyter Notebook, 2020). Data visualization was also made in the same program with the boxplot function.

3. Results and discussion

Indoor farming is the potential solution in supporting nutrition for the growing global population without further converting wild habitats. This system is suitable for farming in urban areas and maximizing the use of vertical space. Indoor farming also allows access to fresh and highly nutritious fruits and vegetables for the urban population, reducing the need for postharvest and transport management. However, the system depends on artificial lighting, which requires optimization to improve specific crops' qualities. This research tested four-light combination treatments based on the RGB ratio percentage, namely 91R/9B, 83R/17B, 47R/53B, 35R/65B, and white fluorescent light as the control (C) (Figure 1). Previous research indicated that light modification in the spectral area of red and blue improved the morphology [27]; [23], yield [27]; [28], and/or antioxidant content [29]; [27]; [23] either in leafy vegetables or fruits. Here, we tested wheatgrass ten days after sowing (DAS) and cultivated it in the treatments mentioned above to determine the potential of providing an antioxidant source in a limited urban household area and within a short time.

Our results (**Figure 2A**) showed no significant difference in the height of plants, but distinct patterns of height increase were recorded in each treatment. Treatment 87R/13B resulted in a sigmoid growth wherein the second observation (6th day), there was a height increase of 9 cm, followed by a stable growth. Treatments 83R/17B, 43R/57B, and 35R/65B showed exponential growth, whereas linear growth was found in control. Meanwhile, the highest average of fresh weight (**Figure 2B**) after harvest was detected in treatment 43R/57B but was not significant to treatment 35R/65B or control. Sigmoidal growth occurs when plants experience small growth at the beginning, followed by exponential growth, and eventually ending in a decrease in growth [30]. Exponential growth occurs from the first observation until the last; the plants experience exponential biomass accumulation [31]. Meanwhile, linear growth is indicated by the constant increase in plant growth [32].

DOI: 10.5614/3bio.2022.4.1.4

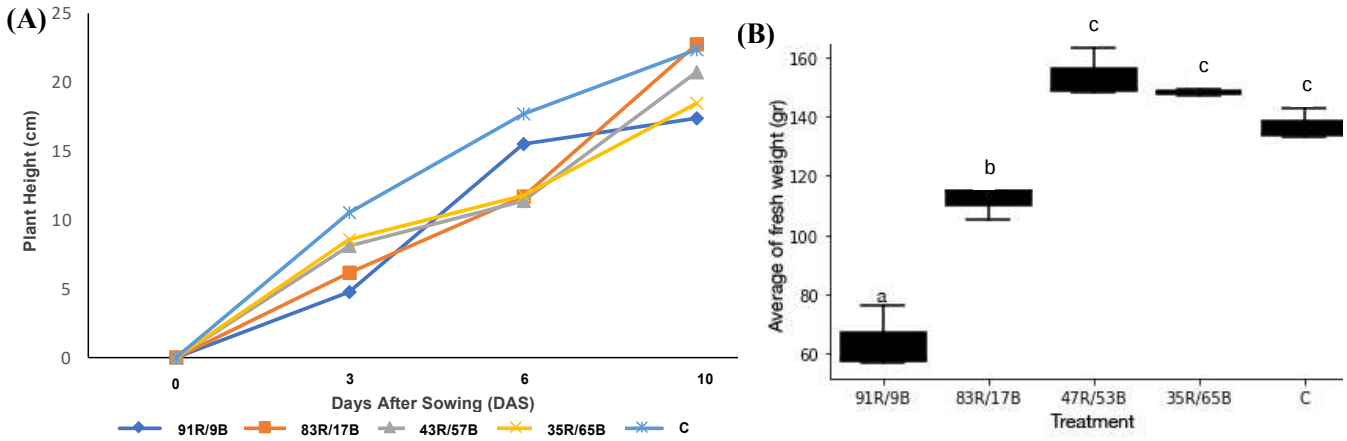

Figure 2. The height (A) and fresh weight (B) of wheatgrass microgreens. Height was recorded on days 3, 6, and 10 after sowing, while fresh weight was only on the day of harvest on 10 DAS. Statistically significant values (Tukey's HSD test, $p<0.05$) are indicated by the letter on top of the box plot corresponding to each treatment.

The light amount and quality directly affect plant growth and chemical composition. Chlorophyll pigments absorb light in the spectral range of red light (663 nm and 642 nm) and blue light (430 nm and 453 nm), affecting plant growth immensely [33]. Red light is perceived by phytochrome receptors and generates responses related to germination, stem elongation, and leaf expansion. Therefore, high-intensity red light applied after germination can accelerate plant height growth in wheatgrass [34]. Blue light stimulates plant growth, leaf expansion, photosynthesis, and pigment accumulation. A combination of blue and red light with a higher ratio of blue light was found to increase fresh mass in comparison to monochromatic light, specifically on lettuce and spinach. However, other plants such as tomatoes and petunias resulted in higher fresh mass when cultivated with intensive red light. This suggests that the effect of red and blue light differs on various species [23]. Despite that, the ratio of red and blue light must be determined carefully because excessive blue light may cause a decrease in fresh mass [33]. Radiation of red light on plant sprouts may maximize photosynthesis. This also eliminates the need for plant protection from photodamage and energy dissipation from other lights. In comparison to a full-spectrum light, radiation with only narrow-length waves on microgreens may decrease the risk of photodamage and increase plant abilities in water absorption and fresh tissue accumulation [19].

Chlorophyll content was also regulated by the spectral area of exposed light, as indicated by the significantly lower content in the leaves cultivated in 91R/9B compared to other treatments and control. A similar result was also recorded by [27] in green leaf lettuce (*Lactuca sativa* L. 'Grand Rapid TBR') that a high ratio of red light reduced chlorophyll content but not significantly when decreased to 87R/13B. They also found that the treatment of blue light dominant showed the opposite effect. However, this was not demonstrated in our research as chlorophyll content in 47R/53B, and 35R/65B treatment were similar to control (Figure 3). This indicates that plant response, in terms of chlorophyll content, to light exposure during cultivation was unique to each species, if not a cultivar. Further, Zheng et al. [34] also reported that blue light exposure improved chlorophyll content in *Sinningia speciosa* but did not in *Cordyline australis* and *Ficus benjamina*.

The antioxidant activity of wheatgrass microgreens was determined by measuring the amount of scavenged DPPH. We found that they were not significantly different among treatments when measured on day 10 after sowing. Shon et al. [27] recorded that the high intensity of red light reduced, while blue light increased antioxidant content in both red leaf lettuce (*Lactuca sativa* L. 'Sunmang') and green leaf lettuce (*Lactuca sativa* L. 'Grand Rapid TBR') after four weeks of treatment. Conversely, increased antioxidant capacity under high-intensity red light treatment was recorded in sprouting (3 days after sowing) lentil, radish, and wheat seeds [23]. Altogether, these suggest that the growth stage of each plant might as well regulate the total antioxidant capacity.

Similar to the other phytochemical content, we found that our results, along with the previous research, could not point to whether red or blue light exposure is more potent to increase carotenoids [34] or flavonoid [27] content. However, in this research, 35R/65B treatment resulted in the highest content of carotenoids, while 87R/17B resulted in the highest flavonoid content. Bohn et al. [34] showed that both high red and blue light intensities increased carotenoid content in *Sinningia speciosa* but not in *Cordyline australis* and *Ficus benjamina*. Also, higher flavonoid content was recorded in red leaf lettuce under high intensity of either red or light treatment, but in green leaf lettuce was only in the plants treated with high intensity of blue light [27].

DOI: 10.5614/3bio.2022.4.1.4

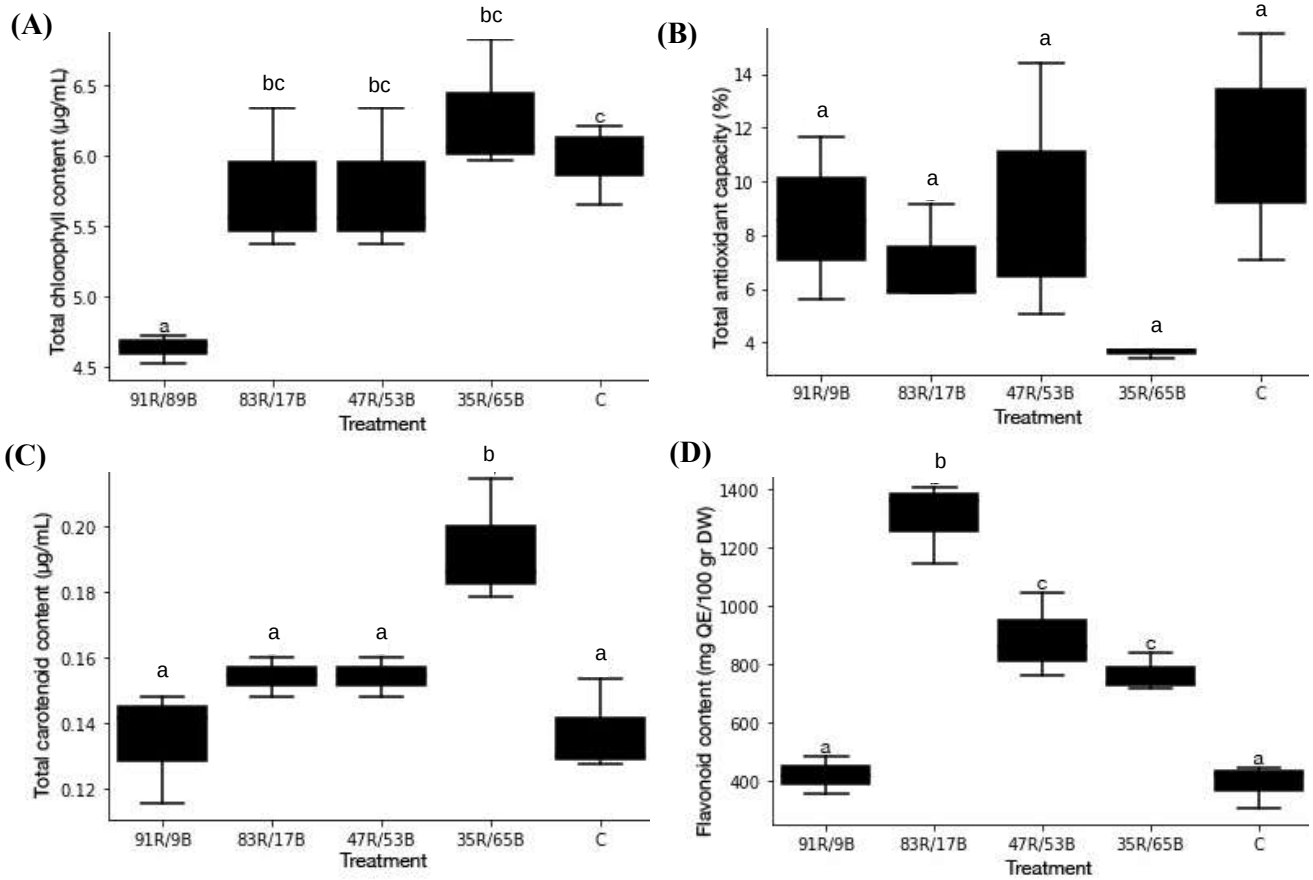

Figure 3. Phytochemical content of the wheatgrass microgreens following 10 days of cultivation in each treatment. Chlorophyll (A) and carotenoids (B) content were determined from identical leaves samples, while total antioxidant capacity (C) and flavonoid content (D) were determined separately. Statistically significant values (Tukey's HSD test, $p<0.05$) are indicated by the letter on top of the box plot corresponding to each treatment.

Even though none of the treatments we tested was able to increase the total antioxidant capacity, we found that 35R/65B and 87R/13B were able to increase carotenoids and flavonoid content, respectively. Carotenoids and flavonoids are among the most sought Phyto-antioxidants due to their health benefits. For instance, as extensively reviewed by [35], dietary intake of carotenoids has been associated with a reduced risk of several chronic diseases, including brain-related diseases, obesity, cardiovascular diseases, type 2 diabetes, and some types of cancer. Whereas routine intake of flavonoids has improved cardioprotective and hepatoprotective capacities [36], anti-aging and depigmenting effects in dermatological applications [37], and has been associated with lower mortality in cancer patients [38].

Furthermore, the antioxidant property of carotenoids and flavonoids have also been considered to boost recovery from COVID-19 by quenching advanced inflammatory [39]; [10]. Fakhri et al. [40] suggested that a certain carotenoid, such as astaxanthin, with their known roles in anti-inflammatory, antiapoptotic, and autophagy-modulatory activities, could be one of the promising treatments to alleviate the complications of COVID-19. Antiviral activity of siponaxanthin has also been explored, and this carotenoid was shown to reduce infection of SARS-CoV-2 pseudovirus on HEK293 cells overexpressing angiotensin-converting enzyme 2 (ACE2) [41]. The antiviral role of flavonoids against SARS-CoV-2 is also currently receiving more attention. For instance, [42] indicated through molecular docking simulation that Quercetin-3-O-rhamnoside flavonoid is a potential drug to inhibit the function of Chymotrypsin-like protease (3CL pro) of the virus.

In conclusion, the health benefits of carotenoids and flavonoids go beyond their role as antioxidants and potentially as antiviral agents (s). Hence, the system we develop in this research could improve the urban

DOI: 10.5614/3bio.2022.4.1.4

population's access to include high carotenoid and flavonoid diets routinely. However, further research to elaborate on specific carotenoids and flavonoids contained in the wheatgrass microgreens would give us a better view of their detailed roles in our health.

4. Conclusion

Increasing demand for nutrition is inevitable as the global population continues to grow. The conversion of wild habitats into farming land is no longer an option as we have witnessed too many negative outcomes from this practice. On the other hand, the prevalence of chronic diseases in the urban population is also growing due to unhealthy lifestyles, such as the limited consumption of fresh vegetables. This research offers a system for the urban population to produce wheatgrass microgreens in their household, with increased content of carotenoids and flavonoids. These two antioxidants are receiving more attention as potential antiviral agent(s) and their already well-defined roles in quenching inflammatory reaction that often complicates infection or chronic diseases. Further research to define the specific carotenoids and flavonoids contained in the wheatgrass microgreen and the system's design so that it is portable and easy to install would further benefit the urban consumers.

Acknowledgements

The authors would like to thank Riset Peningkatan Kapasitas Dosen 2019, Institut Teknologi Bandung, for the research funding granted to KM.

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DOI: 10.5614/3bio.2022.4.1.4

e-ISSN: 2655-8777 http://journals.itb.ac.id/index.php/3bio

Review Article

Heterologous Production of Human Papillomavirus L1 Capsid Protein: Systematic Review and Meta-analysis

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Received: 2021-11-22
Accepted for publication: 2022-03-22

Abstract

The coverage of HPV vaccination in Indonesia remains low due to the high-cost vaccination. The vaccine prices were affected by the production rate of L1, the active substance of HPV vaccines. L1 has been produced using various organisms with varying L1 production rates and immunogenicity. A systematic review and meta-analysis were conducted to determine the organism producing L1 with the highest production, treatments affecting the L1 expression rate, and immunogenicity (represented by anti-L1 IgG titer in mice). The data of L1 titer, induction period, and IgG titer were extracted from 19 articles that have passed the articles screening. The L1 titer and induction period data were used to calculate the L1 production rate, while the IgG titer was used in the immunogenicity analysis. On a 95% confidence level, the meta-analysis revealed weak evidence that *E. coli* produced L1 at the highest rate. The highest IgG titer was induced using L1 expressed in *Saccharomyces cerevisiae*, albeit insufficient evidence on 95% confidence level. Pearson's correlation analysis showed that the concentration of glucose, IPTG, NH_4^+, K^+, Ca^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}, $B_4O_7^{2-}$, $H_2PO_4^-$, HPO_4^{2-}, $Mo_7O_{24}^{6-}$, and citric acid had a positive correlation with L1 production rate in *E. coli*. The treatment injection doses positively correlated with IgG titer in *S. cerevisiae*. This study reveals the mineral salts as the potential treatments to increase L1 production rates.

Keywords: HPV, L1, L1 production rate, anti-L1 IgG titer, Pearson's correlation analysis

1. Introduction

Cervical cancer remains one of the leading causes of mortality in Indonesia. Global Cancer Observatory: Cancer Today (gco.iarc.fr/today) estimated that in 2020, Indonesia would be the third leading country with the highest cervical cancer incidences — with 36,633 cases with 21,003 mortalities [1].

Human papillomaviruses (HPV), especially the high-risk types, are known as the leading risk factor for cervical cancer [2]. HPV is transmitted through sexual intercourse. HPV infection occurs at a site of epithelial abrasion. Persistent infection by high-risk types induces progression to carcinoma [3].

This day, HPV infections can be prevented through virus-like particle (VLP)-based immunization. However, HPV vaccination coverage in Indonesia remains low. Even though high HPV vaccination coverage has been attained in several cities by 2018, it is not the case for other regions, especially rural areas [4]. HPV vaccination costs have soared since Indonesia stopped the funding scheme under the cooperation with Global Alliance for Vaccines and Immunizations (GAVI) for the national HPV vaccination in 2019. Thus, HPV vaccines have become more unaffordable for most Indonesian citizens of a lower-middle-income class.

HPV vaccines contain the HPV virus-like protein (VLP) consisting of solely major capsid protein L1 as the active substance [5]. Up until now, the L1 protein has been produced through the expression of the L1 gene using the cells of various organisms [6-10], and the L1 expression rates vary across different kingdoms of life [7, 11]. Moreover, previous experiments on heterologous L1 expression demonstrated that varying nutrient-rich media composition led to varying L1 production rates [12]. Thus, the choice of organism and media formulation are the decisive factors in building the L1 expression system with a higher L1 production rate. The higher the L1

DOI: 10.5614/3bio.2022.4.1.5

production rate, the lower the selling prices of VLP and, hence, vaccine prices [13]. In addition, the L1 protein produced has to be immunogenic in order to elicit immune responses.

Therefore, this study is aimed to perform a systematic review and meta-analysis to compare the L1 production rate using various organisms and immunogenicity. We also investigated the treatments affecting L1 production rate and immunogenicity.

2. Methodology

2.1. Scope and Search Strategy

The literature search was conducted on September 7, 2021, through PubMed as the search engine. The search terms used were: "human papillomavirus," AND "L1" AND "expression." Only the articles in English were included in this systematic review.

2.2. Inclusion and Exclusion Criteria

Firstly, we screened all the articles based on the titles and abstracts and excluded articles unrelated to the heterologous expression of L1. After that, we performed the second stage of screening and included articles containing the data on L1 titer and induction period. We excluded articles that did not include one or two data mentioned above. Articles of co-expressions and chimeric expressions were also excluded, except for the co-expressions of L1 and the chaperone proteins, since those co-expressions increased the L1 titer.

2.3. Meta-analysis

In this study, the immunogenicity is represented by the post-injection anti-L1 IgG titer in mice (*Mus musculus*). The data of L1 titer, induction period, and anti-L1 IgG titer were extracted from 19 articles that have passed the articles screening.

2.4. Calculation of L1 Production Rate

The L1 titer and expression induction period data were extracted from the final selected articles. We calculated the L1 average production rates according to Equation (1).

$$L1\ average\ production\ rate = \frac{L1\ titre}{expression\ induction\ period} \quad (1)$$

2.5. Hypothesis Testing

We performed Student's *t*-test using Minitab 17 to determine the statistical significance in comparison of the mean L1 average production rate and the mean anti-L1 IgG titer between each organism.

2.6. Determination of Treatments Affecting L1 Production Rate and Anti-L1 IgG Titer

To seek the treatments affecting the L1 production rate and anti-L1 IgG titer, we determined the Pearson's correlation coefficient for the correlation between each treatment with L1 production rate and anti-L1 IgG titer using Minitab 17.

3. Results and discussion

3.1. Articles Screening

The search yielded 891 papers (1983 to 2021) (Figure 1). The first screening based on titles and abstracts excluded 768 articles, leaving 123 articles. Following the second screening based on the data availability, 104 articles were excluded. Ultimately, the selected 19 articles were used in the meta-analysis.

3.2. Characteristics of Selected Articles

The selected articles were categorized according to the organisms used as the expression hosts (Table 1). The organisms include *Escherichia coli* [6, 14-16], *Saccharomyces cerevisiae* [7, 17-19], *Pichia pastoris* [8, 20], baculovirus-infected *Spodoptera frugiperda* [7, 21-25], *Drosophila* [26], Vero cell [9], MRC-3 cell [9] and tobacco (*Nicotiana benthamiana*) [10].

3.3. Production Rate

We extracted 43 data on L1 titer and expression induction period from the selected articles. We calculated each organism's mean L1 average production rate and summarized them in Figure 2. As illustrated in Figure 2, *E. coli* were capable of producing L1 with the highest rate, even though the obtained data were insufficient to support this claim at 95% confidence level due to the high variability of L1 average production rate using *E. coli* (Supplementary Figure 1 and 2). Pearson's correlation analysis for the correlation between 18 treatments and L1 production rate (Supplementary Table 1) revealed that the concentration of glucose, IPTG, NH_4^+, K^+, Ca^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}, $B_4O_7^{2-}$, $H_2PO_4^-$, HPO_4^{2-}, $Mo_7O_{24}^{6-}$ and citric acid had a positive correlation with L1 production rate in *E. coli* (R = 0.820 (P < 0.005), except for IPTG, to which had a correlation coefficient of R = 0.619 (P < 0.005)). Those were the components of the R/2 medium used by Bang *et al*. [6]. Bang *et al*. successfully produced 4,6 g/L of L1 capsid using *E. coli* within 4 h expression induction period on this medium, and it was the highest L1 expression rate recorded in this systematic review.

DOI: 10.5614/3bio.2022.4.1.5

Table 1. Articles classification based on organisms used in experiments.

Organism	Number of Articles
Escherichia coli	4
Saccharomyces cerevisiae	5*
Pichia pastoris	2
baculovirus-infected *Spodoptera frugiperda*	6*
Drosophila	1
mammalian cells	1
Nicotiana benthamiana	1

* One of the articles compares the L1 expression performance of two organisms, including *S. cerevisiae* and baculovirus-infected *S. frugiperda*.

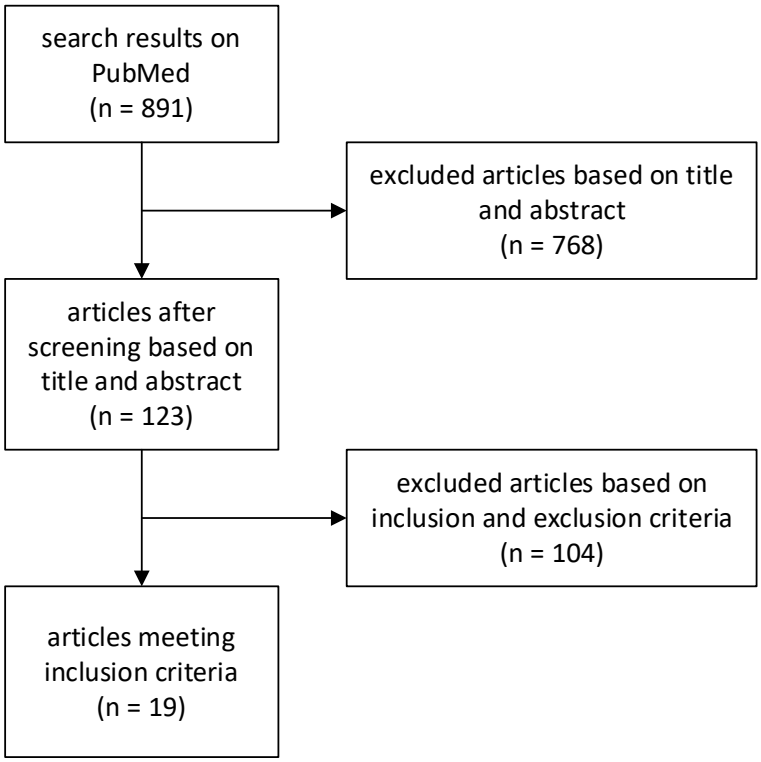

Figure 1. Summary of articles screening.

The L1 production, which attained the highest L1 production rate, is the only study that used the mineral-rich R/2 medium. Bang *et al*. used *E. coli* as the L1 expression host in their study. None of the other studies used similar mineral-rich media as this study. Thus, it is thought that adding the mineral salts into the growth media may escalate the L1 production rates. The mineral salts have been demonstrated to play an essential role in promoting cellular growth. K^+ and Mn^{2+} are the cofactors for several enzymes involved in glycolysis. Ca^{2+} induces the assembly of FtsZ proteins to form protofilaments [27]. The protofilaments can assemble into a Z-ring which drives the membrane invagination during cell division [28]. Fe^{2+} is a ligand for cytochrome and a precursor for Fe-S proteins. Both of those proteins play an essential role in the electron transport chain in cellular respiration. Zn^{2+} was reported to have a major role as the component of the DNA-binding domain on DNA primase [29]. $B_4O_7^{2-}$ is known as the ligand of autoinducer-2 (AI-2) protein which plays a role in quorum sensing [30] to induce biosynthesis of flagella, chemotaxis, and biofilm formation [31]. $H_2PO_4^-$ and HPO_4^{2-}, aside from being the pH buffer, are needed in the biosynthesis of nucleic acids and phospholipids [32]. $Mo_7O_{24}^{6-}$ is the molybdenum (Mo) source. Mo serves as a ligand for nitrate reductase cofactor, which plays an important role in catalyzing anaerobic nitrate reduction into ammonia [33].

DOI: 10.5614/3bio.2022.4.1.5

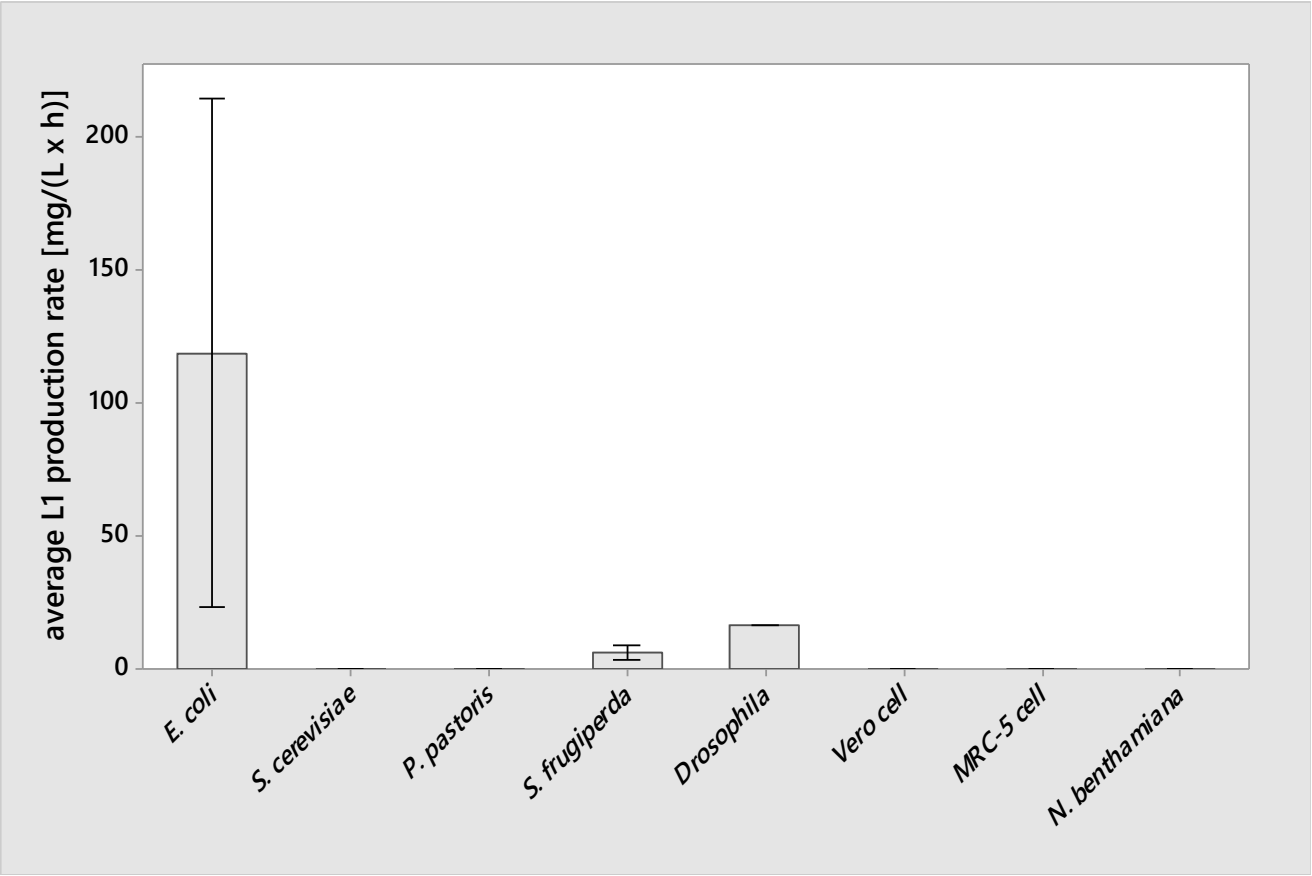

Figure 2. Average L1 production rate for each organism. Bars show the mean of average L1 production rate (not shown for the organism with single datum). Interval bars show the standard deviation. Number of data: *Escherichia coli* (*n* = 12), *Saccharomyces cerevisiae* (*n* = 17), *Pichia pastoris* (*n* = 3), baculovirus-infected *Spodoptera frugiperda* (*n* = 6), *Drosophila* (*n* = 1), Vero cell (*n* = 1), MRC-5 cell (*n* = 1), *Nicotiana benthamiana* (*n* = 2).

Based on this finding, we could suggest the potential idea of implementing the mineral-rich media in L1 production using other organisms. Enriching the media with mineral salts could promote cell viability and thus increase the L1 expression rate using organisms other than *E. coli*. This potential has to be explored further in other organisms.

3.4. Anti-L1 IgG Titer

We extracted 18 data of anti-L1 IgG titer from the selected articles. The mean of anti-L1 IgG titer for each organism was calculated and summarized in Figure 3. As shown in Figure 3, the L1 protein expressed by *Saccharomyces cerevisiae* induced the highest mean of anti-L1 IgG titer in mice, albeit the weak evidence at 95% confidence level [12, 19]. In heterologous L1 expression using *S. cerevisiae*, galactose was added to the media as the inducer [7, 12, 17-19]. However, aside from inducing the heterologous expression, galactose could be utilized as the building block of oligosaccharides in protein glycosylation [34, 35]. In *S. cerevisiae*, the expressed protein is glycosylated [36], and this might alter the immunogenicity of expressed L1 protein [37]. However, injection doses still became the main factor that affected the immunogenicity, as suggested by Pearson's correlation analysis for 3 treatments (glucose concentration, galactose concentration, and doses) and anti-L1 IgG titer (Supplementary Table 2). The correlation analysis revealed a positive correlation of injection doses with anti-L1 IgG titer (R = 0.814 (P < 0.005) in *S. cerevisiae*. Although there is evidence that was varying glucose and galactose concentrations led to different anti-L1 IgG titer [12], in this meta-analysis, further observation on scatter diagrams (Supplementary Figures 4 and 5) indicated a weak potential relationship between glucose concentration and anti-L1 IgG titer as well as galactose concentration and anti-L1 IgG titer (P > 0.05). Furthermore, the P-value suggested insufficient data available to conclude the relationships. This study calls for more experiments investigating the effect of glucose and galactose on the glycosylation performance and thus anti-L1 IgG titer.

DOI: 10.5614/3bio.2022.4.1.5

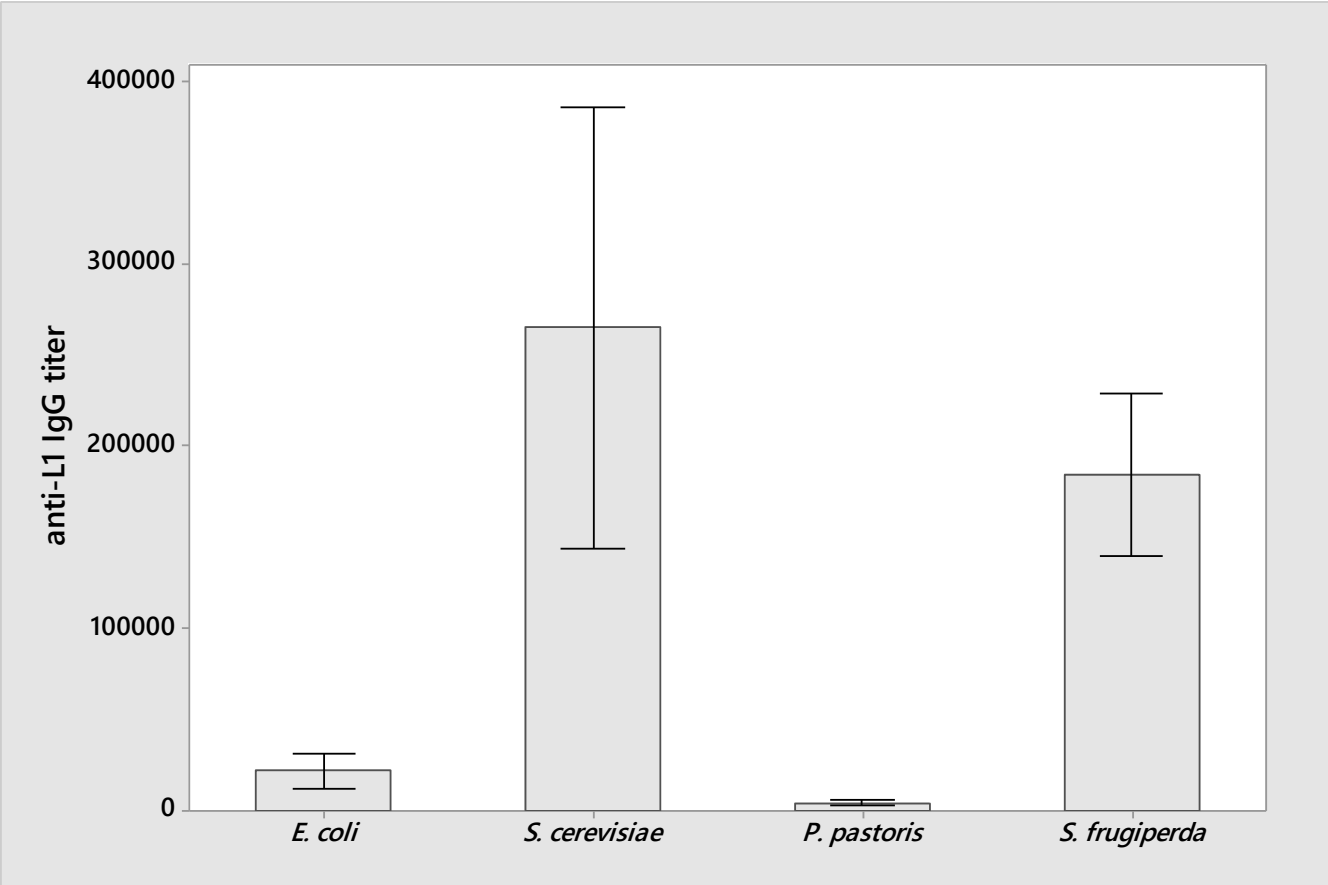

Figure 3. Anti-L1 IgG titer induced by L1 from each organism. Bars show the mean of IgG titer. Interval bars show the standard deviation. Number of data: *Escherichia coli* (*n* = 2), *Saccharomyces cerevisiae* (*n* = 10), *Pichia pastoris* (*n* = 2), baculovirus-infected *Spodoptera frugiperda* (*n* = 4).

4. Conclusion

This is the first study to perform a systematic review and meta-analysis on heterologous L1 expression rate in various organisms and its immunogenicity. The data gathered through this systematic review has yet to provide strong evidence for the statistical significance of different L1 production rates and IgG titers. Meta-analysis revealed weak evidence that *E. coli* produced L1 with the highest rate at 95% confidence level. The highest mean IgG titer was observed on the L1 gene expressed by *Saccharomyces cerevisiae*, albeit the weak evidence at 95% confidence level and its dependence on immunization doses. Pearson's correlation analysis showed that the concentration of glucose, IPTG, NH_4^+, K^+, Ca^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}, $B_4O_7^{2-}$, $H_2PO_4^-$, HPO_4^{2-}, $Mo_7O_{24}^{6-}$, and citric acid had a positive correlation with L1 production rate in *E. coli*, and in *S. cerevisiae*, injection doses had the positive correlation with IgG titer. Additionally, this work provides evidence of the potential role of mineral salts in heterologous L1 expression.

Supplementary

Supplementary material for correlation analysis is provided along with this manuscript.

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DOI: 10.5614/3bio.2022.4.1.5

e-ISSN: 2655-8777 http://journals.itb.ac.id/index.php/3bio

Preliminary Economic Potential Evaluation of Seaweed *Gracilaria* sp. Biomass Waste as Bioindustry Feedstock Through a Biorefinery Approach: A Case Study in Karawang, Indonesia

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Received: 2022-01-25
Accepted for publication: 2022-04-09

Abstract

Seaweed processing usually produces biomass waste from unused and rejected materials, which have economic potential due to their bioactive components. This preliminary study aims to evaluate the economic potential of seaweed biomass waste through a biorefinery approach. Seaweed *Gracilaria* sp. biomass waste samples were collected from a representative seaweed production unit in Karawang, Indonesia, and their biochemical composition was analyzed. Relevant information related to seaweed biomass waste was gathered. The preliminary economic evaluation was assessed from the gross revenue of the proposed products, based on the assumed annual seaweed biomass waste productivity, biochemical composition, and estimated market price of the products. The present study revealed that the dry weight of *Gracilaria* sp. biomass waste contains 63.2% carbohydrates, 13.6% proteins, 1.6% lipids, and 21.5% ashes. Heavy metals were detected in the biomass waste, although no pigments were detected. This study estimates that 52 metric tons/year of *Gracilaria* sp. biomass waste could generate amino acids, fatty acids, lipids, carbohydrates, and minerals with potential gross revenue of ± USD 222,924.6/year. This study suggests that *Gracilaria* sp. biomass waste could be potentially used as feedstock to improve its economic value for bioindustry purposes.

Keywords: Cascading biorefinery, *Gracilaria* sp., Indonesia, Sustainability, Waste nutrient recovery

1. Introduction

Seaweeds are marine commodities that are commonly processed into dried forms or hydrocolloid extracts (e.g., agar, carrageenan, and alginate) for food industry application. Seaweeds are also potential feedstocks for biofuel, nutraceuticals, cosmetics, and other valuable products, due to their bioactive components, such as polyunsaturated fatty acids (PUFAs), pigments, vitamins, minerals, sterols, phenolics, and terpenoids. Those bioactive compounds potentially possess antibacterial, anti-inflammatory, antioxidant, and anti-cholesterol that could treat various health issues, such as hypertension, hyperlipidemia, thrombosis, obesity, diabetes, tumor, and cancer [1, 2]. Furthermore, seaweeds have a high growth rate and photosynthesis efficiency with low lignin content, making seaweed biomass processing into products more straightforward. Seaweed cultivation also does not require freshwater or fertilizer, and it does not compete with agricultural land [1, 3].

Indonesia is the second globally largest seaweed producer, with a productivity of 11.63 million metric tons

DOI: 10.5614/3bio.2022.4.1.6

in 2015 [4]. Karawang Regency is a seaweed cultivation center in Indonesia, particularly red seaweed *Gracilaria* sp., with a productivity of 750.05 metric tons in 2020 and 60 seaweed cultivation business units in 2021 [5, 6]. Karawang also acts as a hub to collect seaweeds from the neighboring areas (e.g., Bekasi, Subang, Indramayu) and process them into dried forms. The dried seaweeds are traded to nearby agar industries, such as PT. Agarindo at Tangerang [7].

However, most seaweed industries in Indonesia, including Karawang, still focus on processing a single product (e.g., agar from *Gracilaria* sp.), which generates a lot of residual waste [5]. Furthermore, the biomass that does not meet the standard quality or rejected biomass is also considered waste [1]. The waste is traditionally used as a supplementary feed for the nearby fish ponds and construction materials mixture that is low in economic value or discarded, creating environmental issues [8]. On the other hand, hypothetically, seaweed biomass waste still contains bioactive compounds that can generate more valuable products [2].

The seaweed biomass waste could be utilized to generate various high-value products, such as pharmaceuticals, fine chemicals, and biofuel, simultaneously through the biorefinery concept approach. Biorefinery is a production scheme of high-value products by maximizing biomass utilization and revenue, minimizing production cost and waste generation or zero waste [1-3]. Biorefinery application on seaweed biomass waste could contribute to the sustainable development goals (SDGs), including food security, sustainable natural resources usage, environmental impacts reduction, employment creation, and competitiveness reinforcement. However, the biorefinery on seaweed biomass waste is still an emerging approach. Therefore, its development requires identifying potential products and processes, designing the integrated processes, and assessing the economic and life-cycle to ensure the seaweed biomass waste-based industrial activities are optimum and economically feasible [1, 9].

Based on the above background and problems stated, this preliminary study aims to evaluate the economic potential of seaweed *Gracilaria* sp. biomass waste through a biorefinery approach. This case study is based on a representative seaweed production unit in Karawang, Indonesia, using the data on seaweed biomass waste productivity and biomass components. The results of this preliminary study can serve as a base for further advanced laboratory works, techno-economic analysis, and life-cycle assessment. Hopefully, this study could also trigger the development of eco-friendly and bio-based seaweed industries with more diverse valuable products.

2. Materials and Methods

2.1. Seaweed Biomass Waste Sample and Production Data Collection

Seaweed (*Gracilaria* sp.) biomass waste sample was collected in January-February 2021 during the rainy season from the warehouse of Koperasi Mina Agar Makmur, Tirtajaya District, Karawang Regency, West Java, Indonesia (6°00'21"S 107°14'02"E). Koperasi Mina Agar Makmur is a business unit in Karawang engaged in the seaweed processing area. Seaweed *Gracilaria* sp. biomass was identified according to the seaweed identification book [10]. *Gracilaria* sp. has brownish red to yellowish or greenish color, cylindrical thallus (with solid, brittle, cylindrical, to compressed branches), and dichotomously branched. The crude seaweed biomass waste sample was stored in a plastic container at room temperature before further laboratory analysis.

The data on seaweed biomass waste production was gathered through an interview with the head of Koperasi Mina Agar Makmur in January 2021. The collected data comprised seaweed processing steps, seaweed quality parameters, the quantity of weekly dried seaweed shipping, and monthly seaweed biomass waste generated.

2.2. Seaweed Biomass Waste Composition Analysis

Proximate analysis of seaweed biomass waste, comprising moisture content, total proteins, total lipids, ashes, and total carbohydrates, was carried out in Regional Technical Unit Laboratory, Fisheries Product Quality Testing and Implementation, Department of Fisheries and Marine Affairs of West Java, Cirebon, Indonesia. The moisture content, total proteins, total lipids, and ashes were analyzed following the National Standardization Agency of Indonesia described in reference [11-14]. Total carbohydrates were analyzed through a method described by the Association of Official Analytical Chemists [15].

Analysis of fatty acids, amino acids, chlorophylls, β-carotenes, vitamin C, and heavy metals were carried out in the laboratory of PT. Saraswanti Indo Genetech, Bogor, Indonesia. Fatty acids were analyzed using a gas chromatography (GC) protocol according to reference [16]. Amino acids (without cysteine, methionine, tryptophan, asparagine, and glutamine) were analyzed using an ultra-performance liquid chromatography (UPLC) protocol according to reference [17]. Chlorophylls, calculated as cuprum, were analyzed by an inductively coupled plasma-optical emission spectrometry (ICP-OES) protocol according to reference [18]. Vitamin C and β-carotenes were analyzed by a high-performance liquid chromatography (HPLC) protocol according to reference [19, 20]. Heavy metals were

DOI: 10.5614/3bio.2022.4.1.6

analyzed by an inductively coupled plasma-mass spectrometry (ICP-MS) protocol according to reference [21]. All biomass composition was analyzed in duplicate, and the results were presented as mean ± standard deviation.

2.3. Potential Products Identification and Productivity Calculation

The identification of potential products from seaweed biomass waste was carried out through a literature search from April-August 2021. A scholarly web search engine, Google Scholar (https://scholar.google.com), with keywords of ("seaweed+biomass+waste+refinery") and ("red+seaweed+biomass+waste+refinery") was applied for literature search. The literature from the first five pages of the search results, with 3-5 referred websites per page, was used to identify the potential products.

The potential productivity of products generated from seaweed biomass waste was calculated according to Eq. (1).

$$P_{prod} = P_{BW} \times C_{prod,BW} \quad (1)$$

P_{prod} is the potential productivity of the product (kg.year^{-1}), P_{BW} is the productivity of seaweed biomass waste generated (kg.year^{-1}), and $C_{prod,BW}$ is the product content in seaweed biomass waste (% or ppm).

2.4. Economic Potential Evaluation

The gross revenue of the products represented the economic potential of products generated from seaweed biomass waste. The gross revenue of the products was calculated according to Eq. (2).

$$GR_{prod} = P_{prod} \times V_{prod,unit} \quad (2)$$

GR_{prod} is the potential gross revenue of the product (USD.year^{-1}), P_{prod} is the potential productivity of the product (kg.year^{-1}), and $V_{prod,unit}$ is the selling price per unit of product (USD.kg^{-1}). The selling price of products was obtained from an online web store (https://www.alibaba.com), accessed on October-November 2021.

3. Results and discussion

In this section, the productivity of seaweed biomass waste generation from the representative seaweed production unit in Karawang, Indonesia, will be presented. Then, the seaweed biomass waste composition will be displayed and discussed regarding the potential products made from the composition. Finally, the potential economic value of the products will be evaluated using information from the previous sub-sections.

3.1. Seaweed Biomass Waste Generation

Koperasi Mina Agar Makmur has the main business activity of pressing dried seaweed collected from seaweed farmers and trading the products as the feedstock for agar industries. The process flow diagram of seaweed pressing steps is shown in Figure 1. The average monthly seaweed collected from the seaweed farmers is 120 metric tons (flow 1). During the sortation step, the sorting of seaweed biomass is based on the quality, which is reflected by appearance (dark, not exposed to rain or whitish brown), morphology (long or short thallus), and aroma (product-specific, fresh or poor). Seaweed biomass waste is generated from the drying step (flow 6), in which the seaweed biomass is sun-dried. The generated waste from the drying step contains broken and wet seaweed due to exposure to rain. Additionally, seaweed biomass waste is also generated from storage 2, weighing 2, and pressing and packaging steps (flow 10, 12, and 14), containing broken seaweed. Seaweed biomass wastes from flow 6, 10, 12, and 14 were not sorted and kept in the warehouse before further handling. The average weekly dried and pressed seaweed shipped to the agar industry (flow 17) is 18 metric tons, while the average weekly seaweed biomass waste generated is one metric ton [8]. Assuming that one year equals 12 operational months and 52 working weeks, the overall annual pressing unit processes 1,440 metric tons of seaweed feedstocks from the farmers to produce 936 metric tons of dried and pressed seaweeds, with the seaweed biomass waste generated of 52 metric tons.

3.2. Seaweed Biomass Waste Composition and Potential Products

The proximate composition of seaweed *Gracilaria* sp. biomass waste, which consisted of total proteins, total lipids, total carbohydrates, ash, and moisture content, is shown in Table 1. The characterization of seaweed biomass waste composition is essential to identify potential products that can be generated and its biorefinery strategy [2].

3.2.1. Proteins and Amino Acids

The protein content in the seaweed biomass waste was 13.65 ± 0.02% DW. This value is within the range of seaweed protein content from the literature, 0.60 - 45.00% DW (Table 1), which is broad. The broad seaweed protein content could occur due to species, habitat, and seasonal variation [2]. Nonetheless, previous studies have indicated that seaweeds are high in protein content, comparable to the protein content in eggs, chickens, beef, fish, milk, and grains. Notably, red seaweed such as *Gracilaria* sp. has higher protein digestibility than green and brown seaweed [1, 2, 9, 22, 23, 25, 29, 30].

DOI: 10.5614/3bio.2022.4.1.6

Table 1. The proximate composition of *Gracilaria* sp. biomass waste.

Parameters	*Gracilaria* sp. biomass waste*	Literature* [1, 7, 22-28]
Total proteins (%)	13.65 ± 0.02	0.60 - 45.00
Total lipids (%)	1.65 ± 0.42	0.30 - 7.10
Total carbohydrates (%)	63.20 ± 0.50	3.80 - 78.70
Ash (%)	21.51 ± 0.06	7.36 - 40.30
Moisture (%)	22.00 ± 0.25[#]	4.53 - 11.91[#]

Notes:
*Based on dry weight (DW)
[#]Based on fresh weight (FW)

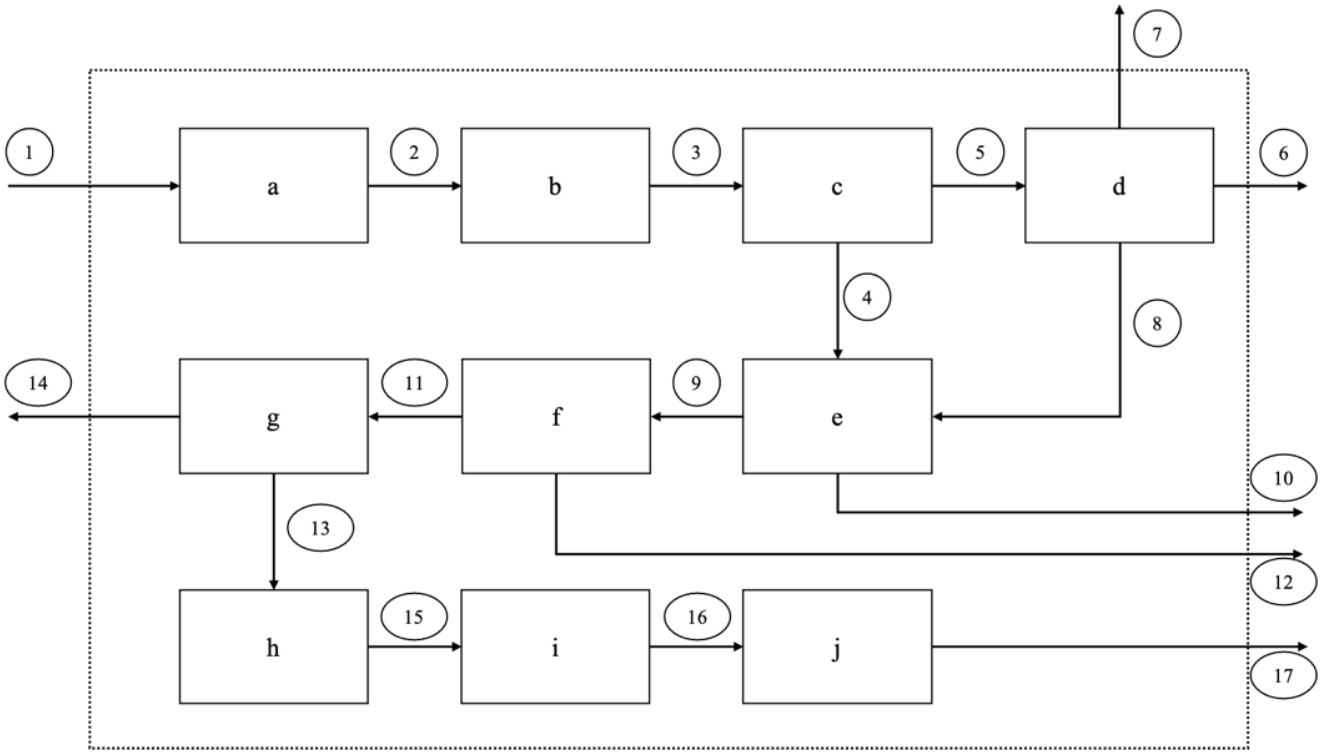

Figure 1. The process flow diagram of seaweed pressing steps in Koperasi Mina Agar Makmur. Unit operations: a) Storage 1 (t = 5 h); b) Weighing 1 (t = 1 - 2 h); c) Sortation (t = 1 h); d) Drying (t = 2 h); e) Storage 2 (t = 5 h); f) Weighing 2 (t = 15 min); g) Pressing and packaging (t = 10 - 20 min); h) Labelling and coding (t = 10 min); i) Storage 3 (t = 3 - 4 d); j) Loading and shipping (t = 5 - 7 h). Material flows: Flow 1 - 5, 8, 9, 11, 13, 15 - 17 represent main seaweed pressing materials; Flow 6, 10, 12, and 14 represent seaweed biomass waste; Flow 7 represents water vapour [8].

With the protein content, seaweed biomass waste can still be potentially used as an alternative protein source as a whole form or as an extract. Seaweeds' protein also has several biological activities, such as antibacterial, antioxidant, immunostimulant, anti-thrombotic, anti-inflammatory, anti-hypertension, and anti-coagulants, which can be applied as pharmaceutical, nutraceutical, and cosmetic products [1, 9, 22, 23, 25, 26, 29, 31]. Some protein recovery methods have been reported for seaweed biorefinery purposes. Cian et al. [32, 33] recovered protein from *Porphyra columbina* biomass through sequential steps of size reduction, centrifugation, filtration, and enzymatic hydrolysis (e.g., acid protease and exoprotease). Baghel et al. [34] obtained the protein extract from *Gelidiella acerosa* and

DOI: 10.5614/3bio.2022.4.1.6

Gracilaria dura biomass through aqueous extraction by phosphate buffer, centrifugation, and purification with ammonium sulfate. Consecutive steps of centrifugation, separation through ion exchange resins, and purification through dialysis also has been reported [26].

The profile of amino acids, which are the building blocks of protein, in the seaweed biomass waste is shown in Table 2. All analyzed amino acids were within the range that has been reported in the literature. The seaweed biomass waste also contained non-essential, particularly essential amino acids, which are important for metabolism [9, 26, 29, 35]. As can be seen in Table 2, L-leucine and L-histidine was the highest and lowest essential amino acid (8.21 ± 0.02 and 1.10 ± 0.01 mg/g, respectively). In comparison, L-glycine and L-tyrosine was the highest and lowest non-essential amino acid (8.98 ± 0.03 and 3.53 ± 0.00 mg/g, respectively) in the seaweed biomass waste. Different types of amino acids have unique prospective functionality. For instance, L-leucine is an essential amino acid to regulates important metabolic networks for growth, immunity, maintenance, and reproduction [36]. L-lysine is an essential amino acid for monogastric livestock's feed which is rarely found in terrestrial plants [9, 30]. L-glycine is a non-essential amino acid that has a role in sleep and memory [26]. L-glutamic acid is a non-essential amino acid with an umami taste characteristic that can be used as an additive in foods [2, 23, 25, 26, 37]. L-glutamic acid can also be used as a feedstock to produce high-value chemicals, such as N-methyl pyrrolidone, N-vinylpyrrolidone, and acrylonitrile [38]. Due to protein content and variability of amino acids, seaweed biomass waste is prospective to be used as a source for protein/amino acids biorefinery. However, biorefinery strategies that maintain protein/amino acids' structure and functionality must be considered. Moreover, amino acids production requires extra steps and thus extra cost [2, 26].

3.2.2. Lipids and Fatty Acids

The seaweed biomass waste sample in this study had 1.65 ± 0.42% DW of lipid content. The lipid value was within the range reported in the literature, 0.30 - 7.10% DW (Table 1). Generally, seaweeds have low lipid content [1, 2, 9, 22, 23, 25, 30, 37]. Due to the low lipid content, seaweeds can be used as a low-calorie food source [25]. Seaweeds' lipids are also composed of unsaturated and saturated fatty acids, as shown in Table 3 [1, 2, 9, 23, 25].

The high proportion of saturated fatty acids compared to unsaturated fatty acids in the seaweed biomass waste (Table 3) was similar to seaweeds' fatty acids proportion reported elsewhere [25, 30]. The fatty acid analysis results showed that palmitic acid (C16:0, 49.20 ± 0.35% of total fatty acids) and stearic acid (C18:0, 9.13 ± 0.12% of total fatty acids) dominated the saturated fatty acids proportion in *Gracilaria* sp., which agreed with the literature [9, 23]. However, in contrast to the reported literature, unsaturated omega-3 fatty acids, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), were not detected in this biomass. The difference in fatty acids proportion in this study and from the literature could occur due to environmental factors, processing conditions, or the content value below the minimum detection level of the method. Environmental factors include geographical location, climate, light intensity, salinity, temperature, nutrition availability, seaweed age, and harvesting time. Processing conditions, including temperature, humidity, and storage, could degrade fatty acid content [22, 23, 25, 30]. In this study, the seaweed biomass waste is generated after around 15 hours of the process (from storage 1 to weighing 2 steps), mostly performed at room temperature. The duration and temperature of the previous steps might degrade some of the fatty acid content, including DHA and EPA, in the sample. Nevertheless, the seaweed biomass waste could be used as feedstocks in food, feed, cosmetics, biotechnology, and pharmaceutical sectors due to its fatty acids content [25]. Fatty acids can also be used as raw materials to manufacture biomaterials, industrial chemicals, and biofuel [38]. The lipid content could be recovered by a combination step of chloroform-methanol extraction, centrifugation, decantation, filtration, and evaporation as reported by [34] from *G. dura* biomass. Billakanti et al. [39] recovered lipids containing PUFAs from *Undaria pinnatifida* biomass with a combination step of enzyme pre-treatment, centrifugation, dimethyl ether and ethanol co-extraction, and evaporation. Further development of lipids recovery for biorefinery purposes must maintain the stability of the lipids/fatty acids in various conditions [9].

3.2.3. Carbohydrates

The seaweed biomass waste had a carbohydrate content of 63.20 ± 0.50% DW, which is within the value range of red seaweed carbohydrate in the literature, 3.80 - 78.70% DW (Table 1). Like protein, the carbohydrate composition in seaweed is broad due to species, environmental, and processing variations [30]. Various studies have indicated high carbohydrate content in seaweeds, with cellulose as one of the major carbohydrate classes in red seaweed.

Cellulose is the main constituent of the seaweed cell wall comprised of glucose unit chains. Besides being used as a dietary fiber source, cellulose can be used as a substrate to produce biofuel (e.g., by alcohol fermentation or anaerobic digestion) [26, 38, 40, 41]. Furthermore, cellulose can also be further processed into cellulose microfibrils, which is valuable in the health sector [26]. Cellulose residues and their derivatives could also be used as feedstocks for building, textile, pulp and paper, pharmaceutical, and food sectors [26, 29]. Several cellulose derivatives used in industrial applications are levulinic acid, 2,5-furandicarboxylic acid (FDCA), and lactic acid, with a potential market value of 3-5 times higher than to bioethanol

DOI: 10.5614/3bio.2022.4.1.6

[26]. Unlike terrestrial plants, seaweeds have low or even no lignin composition, making seaweed biomass processing less complicated by bypassing the delignification step [38].

3.2.4. Ash, Heavy Metals, and Moisture Content

The ash content in the seaweed biomass waste was 21.51 ± 0.06% DW, which is inside the range of seaweed ash values from the literature, 7.36 - 40.30% DW (Table 1). The ash content in seaweeds is higher than in terrestrial plants, generally 5 - 10% DW [2, 23]. Ash content represents minerals and traces elements in the seaweed biomass waste [2, 7, 25, 41]. Seaweeds generally have high mineral composition to withstand the salt-rich nature of seawater. The seaweed cell wall has polysaccharide structures supplemented with functional groups, such as hydroxyl, sulfate, and carboxyl, providing an ion-exchange function. This feature supports seaweeds in absorbing and maintaining minerals from their habitat [2, 7, 23, 25, 29, 30, 41]. Several mineral contents in seaweed biomass are K, Na, Ca, Mg, Fe, Zn, S, Cu, Co, B, and Se. Minerals have a broad scope of functionality, such as controlling hypertension for health purposes and triggering plant growth for agriculture purposes. Due to the mineral content, seaweed biomass waste could be applied for food supplements, animal feeds, and fertilizer [1, 2, 23, 25, 30, 41]. However, seaweeds could also absorb heavy metals from their environment due to their ion-exchange feature. Therefore, it is crucial to assess the heavy metal content before further processing the seaweed biomass waste [25, 26, 30, 41].

In this study, the heavy metals detected in the seaweed biomass waste were cadmium (Cd, 0.05 ± 0.00 mg/kg DW) and lead (Pb, 4.33 ± 0.06 mg/kg DW). Other heavy metals, including mercury (Hg), arsenic (As), and tin (Sn), were not detected (Table 4). The Cd level in the seaweed biomass waste was still under the maximum safety limit, 0.1 mg/kg DW, according to the National Standardization Agency of Indonesia [42]. On the other hand, the Pb level in the seaweed biomass waste exceeded the maximum safety limit, 0.3 mg/kg DW [42]. The heavy metal content could be affected by several factors, such as geographical location, sea wave, temperature, salinity, light intensity, pH, weather, and seaweed age [23, 25, 41]. The safety risk due to heavy metal content in seaweed biomass waste could be reduced by applying desorption media, such as HCl, NaOH, $CaCl_2$, and deionized water to take the heavy metals from the seaweed biomass waste [26].

Table 2. The amino acid composition of *Gracilaria* sp. biomass waste.

Parameters	*Gracilaria* sp. biomass waste*	Literature* [23, 25, 30, 35]
EAAs		
L-leucine (mg/g)	8.21 ± 0.02	0.38 - 19.44
L-phenylalanine (mg/g)	7.49 ± 0.07	1.42 - 14.19
L-threonine (mg/g)	7.07 ± 0.02	1.32 - 20.57
L-valine (mg/g)	6.95 ± 0.02	0.15 - 15.24
L-isoleucine (mg/g)	5.96 ± 0.01	1.22 - 10.51
L-lysine (mg/g)	3.24 ± 0.01	0.22 - 12.08
L-histidine (mg/g)	1.10 ± 0.01	0.18 - 3.43
NEAAs		
L-glycine (mg/g)	8.98 ± 0.03	3.20 - 15.50
L-glutamic acid (mg/g)	8.87 ± 0.02	2.54 - 34.68
L-aspartic acid (mg/g)	8.57 ± 0.01	5.34 - 36.25
L-serine (mg/g)	7.39 ± 0.01	2.23 - 12.87
L-alanine (mg/g)	7.33 ± 0.02	1.46 - 21.11
L-arginine (mg/g)	7.13 ± 0.02	3.33 - 19.97
L-proline (mg/g)	4.32 ± 0.00	0.46 - 14.45
L-tyrosine (mg/g)	3.53 ± 0.00	1.25 - 9.46

Notes:
*Based on dry weight (DW)
EAAs: Essential amino acids; NEAAs: Non-essential amino acids

DOI: 10.5614/3bio.2022.4.1.6

Table 3. The fatty acids composition of *Gracilaria* sp. biomass waste.

Parameters	*Gracilaria* sp. biomass waste*	Literature* [22, 23, 25, 30]
SFAs		
Palmitic acid, C16:0 (%)	49.20 ± 0.35	4.28 - 39.80
Stearic acid, C18:0 (%)	9.13 ± 0.12	0.50 - 2.79
Lauric acid, C12:0 (%)	7.02 ± 0.16	0.18 - 0,20
Myristic acid, C14:0 (%)	3.96 ± 0.06	0.50 - 4.91
Butyric acid, C4:0 (%)	N.D.	0.04
Caproic acid, C6:0 (%)	N.D.	-
Caprylic acid, C8:0 (%)	N.D.	0.11
Capric acid, C10:0 (%)	N.D.	0.04
Undecanoic acid, C11:0 (%)	N.D.	0.01
Tridecanoic acid, C13:0 (%)	N.D.	0.05
Pentadekanoic acid, C15:0 (%)	N.D.	0.09 - 0.85
Heptadecanoic acid, C17:0 (%)	N.D.	0.02 - 0.57
Arachidic acid, C20:0 (%)	N.D.	0.31
Heneicosanoic acid, C21:0 (%)	N.D.	-
Behenic acid, C22:0 (%)	N.D.	0.23 - 2.40
Trichosanoic acid, C23:0 (%)	N.D.	0.07
Lignoseric acid, C24:0 (%)	N.D.	0.26 - 0.30
MUFAs		
cis-oleic acid, C18:1ω9c (%)	24.47 ± 0.07	0.18 - 8.61
Miristoleic acid, C14:1 (%)	N.D.	0.43
Pentadecenoic acid, C15:1 (%)	N.D.	0.03 - 0.07
Palmitoleic acid, C16:1 (%)	N.D.	0.28 - 5.76
Heptadecenoic acid, C17:1 (%)	N.D.	0.43
trans-oleic acid, C18:1ω9t (%)	N.D.	2.87 - 3.89
Eicocyanic acid, C20:1 (%)	N.D.	0.28 - 0.30
Erucic acid, C22:1 (%)	N.D.	0.33
Nervonic acid, C24:1ω9 (%)	N.D.	0.18
PUFAs		
cis-linoleic acid, C18:2ω6c (%)	5.97 ± 0.09	0.59 - 3.20
trans-linoleic acid, C18:2ω6t (%)	N.D.	-
cis-linolenic acid, C18:3ω3c (%)	N.D.	0.14 - 10.90
Eicosadienoic acid, C20:2 (%)	N.D.	0.25
Eicosatrienoic acid C20:3ω3 (%)	N.D.	1.81 - 2.15
AA, C20:4ω6 (%)	N.D.	0.26 - 33.85
EPA, C20:5ω3 (%)	N.D.	1.40 - 1.79
Docosadienoic acid, C22:2 (%)	N.D.	-
DHA, C22:6ω3 (%)	N.D.	0.20 - 48.36
Omega-3 fatty acids (%)	N.D.	-
Omega-6 fatty acids (%)	5.97 ± 0.09	-
Omega-9 fatty acids (%)	24.47 ± 0.07	-
Saturated fatty acids (%)	69.56 ± 0.02	7.53 - 46.90
Unsaturated fatty acids (%)	30.44 ± 0.02	-
Monounsaturated fatty acids (%)	24.47 ± 0.07	19.11 - 38.30
Polyunsaturated fatty acids (%)	5.97 ± 0.09	25.10 - 51.20

Notes:
*Based on total fatty acids
N.D.: not detected; SFA: saturated fatty acid; MUFA: Monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; AA: arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid

DOI: 10.5614/3bio.2022.4.1.6

Table 4. The vitamin, pigments, and heavy metals composition of *Gracilaria* sp. biomass waste.

Parameters	*Gracilaria* sp. biomass waste*	Literature* [23, 25, 30, 35, 43]	Standard* [42]
Vitamin & pigments			
Vitamin C (mg/100 g)	N.D.	2.51 - 1466.00	-
β-carotene (mg/g)	N.D.	0.01 - 18.02	-
Chlorophyll (mg/g)	N.D.	0.01 - 5.74	-
Heavy metals			
Pb (mg/kg)	4.33 ± 0.06	956	< 0.3
Cd (mg/kg)	0.05 ± 0.00	0.3 - 257	< 0.1
Hg (mg/kg)	N.D.	< 10	< 0.5
As (mg/kg)	N.D.	5.8	< 1.0
Sn (mg/kg)	N.D.	-	< 40.0

Notes:
*Based on dry weight (DW)

The moisture content in the seaweed biomass waste was 22.00 ± 0.25% FW, higher than the seaweed moisture content reported in the literature, 4.53 - 11.91% FW (Table 1). Although the seaweed biomass has been dried before the pressing steps, high humidity due to the rainy season and long process duration (around 15 hours) might add more moisture content to the observed sample [8]. High moisture content in the seaweed biomass waste must be avoided since it could accelerate the degradation of bioactive compounds. Additionally, it could also add energy input and cost required to dry the sample before further processing steps [38].

3.2.5. Vitamins and Pigments

Red seaweed, such as *Gracilaria* sp., is known for its various vitamins (vitamin A, B, C, D, E), pigments (chlorophylls, carotenoids, phycobiliproteins), and secondary metabolites that have health-related activities, such as antioxidant and antimicrobial [1, 2, 9, 22, 23, 25, 31, 40, 41]. However, in this study, our samples did not detect the presence of vitamin C, β-carotene, and chlorophyll (Table 4). The vitamin C, (-carotene, and chlorophyll content range in seaweed biomass found in other studies is 2.51 - 1,466.00 mg/100 g, 0.01 - 18.02 mg/g, and 0.01 - 5.74 mg/g, respectively (Table 4). Undetected vitamin and pigment components could occur due to the values below the minimum detection value of the method. The observed seaweed biomass waste was withered and pale-colored, indicating low or no vitamin and pigment content in the sample. The vitamin and pigments composition could be affected by various factors, such as salinity, temperature, weather, processing, and storage conditions [25]. Other analysis methods, such as the ferric reducing ability of plasma (FRAP), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays, could be applied in further studies to assess the presence and activity of the vitamins, pigments, and other secondary metabolites [22].

Assessing seaweed biomass waste composition is the initial step of products development based on the biorefinery approach, which will support identifying the products and processing steps that will be applied [38]. However, the seaweed biomass waste composition is unstable due to species, environmental, and processing factors, as previously discussed. Therefore, biorefinery-based methods that are flexible on these variations should be addressed in future development [30].

3.3. Productivity and Economic Potential Evaluation

Based on the annual productivity information (52 metric tons/year) and composition assessment of the seaweed biomass waste in this study (Table 1-4), the productivity and economic potential of the products from the seaweed biomass waste are presented in Table 5. For the record, this evaluation has only applied gross revenue calculation. The process efficiency factors and process/equipment-related costs have not been included, and the more specific techno-economic analysis will be addressed in the subsequent studies. As shown in Table 5, the price per unit of products was mostly obtained from an online website [44], using the average price of industrial-grade products. The price for the unassessed amino acids came from the average price of overall amino acids. The other lipids productivity came from subtracting total lipids content (Table 1) with the detected fatty acids (Table 3). Possibly, the other lipids were glycerols and lipid-like metabolites, such as sterols, terpenoids, and tocopherols, which are not broken down into fatty acids [2]. The price for the other lipids came from the price of glycerols. Additionally, the price per unit of carbohydrate and mineral (represented by fertilizer price) was obtained from [28, 45]. Hence, 52 metric tons/year of seaweed biomass waste could potentially generate 5,534.93 kg/year of amino acids (USD 132,095.28), 51.71 kg/year of fatty acids (USD 1,540.99), 616.49 kg/year of other lipids (USD

DOI: 10.5614/3bio.2022.4.1.6

616.49), 25,632.70 kg/year of carbohydrates (USD 56,391.94), and 8,724.30 of minerals (USD 32,279.91). Water (11,439.87 kg/year) and heavy metals (0.18 kg/year) were not economically evaluated. Thus, the potential annual gross revenue of products generated from seaweed biomass waste was USD 222,924.60. It means that the economic value of a kilogram of seaweed biomass waste could potentially be increased from USD 0.00 to USD 4.29 through the biorefinery approach.

The application of the biorefinery approach on the seaweed biomass waste could be made by implementing the cascading processes. Cascading processes focus on generating products with higher economic value first based on the seaweed biomass waste composition, followed by lower economic value products. With this approach, the seaweed biomass waste valorization would be more efficient and generate economic value enhancement of the products [1, 2, 46]. From higher to lower economic, the product leveling are pharmaceuticals/fine chemicals, nutraceuticals, cosmetics, foods, feeds, fertilizers/biostimulants, and energy [2, 26, 46]. According to that classification, the biorefinery of seaweed biomass waste could start from amino acids/proteins (for pharmaceutical, nutraceutical, cosmetic, food sectors), fatty acids/lipids (for pharmaceutical, nutraceutical, cosmetic sectors), fertilizer (from minerals and biomass residue, for agriculture sector), and bioethanol (from carbohydrate, for the energy sector). By incorporating the processing strategies from [26, 29, 32-34, 39], the proposed design of the hypothetical biorefinery process of the seaweed biomass waste is shown in Figure 2.

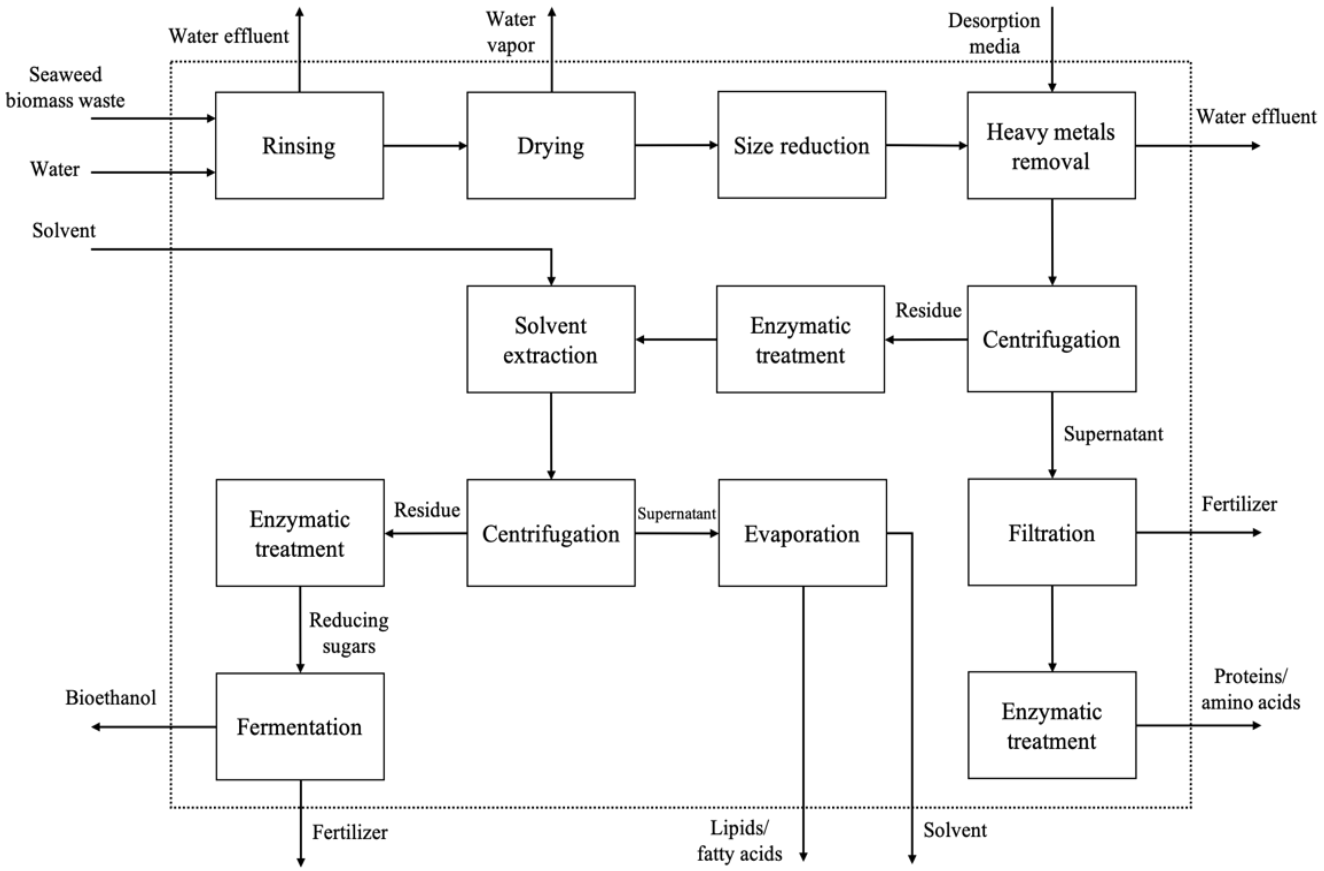

Figure 2. The preliminary hypothetical biorefinery process scheme of seaweed biomass waste.

DOI: 10.5614/3bio.2022.4.1.6

Table 5. The productivity and economic potential of products from seaweed biomass waste.

Products	Annual productivity potential (kg.year^{-1})	Price per kilogram (USD.kg^{-1}) [44-46]	Annual gross revenue (USD)
Amino Acid Total	5,534.93		132,095.28
L-histidine	57.13	50.00	2,856.50
L-isoleucine	309.95	40.00	12,398.00
L-leucine	427.02	20.00	8,540.40
L-lysine	168.63	20.00	3,372.60
L-phenylalanine	389.28	15.00	5,839.20
L-threonine	367.76	20.00	7,355.20
L-valine	361.28	30.00	10,838.40
L-alanine	381.31	30.00	11,439.30
L-arginine	370.90	10.00	3,709.00
L-aspartic acid	445.83	40.00	17,833.20
L-glutamic acid	461.12	20.00	9,222.40
L-glycine	467.21	10.00	4,672.10
L-proline	224.89	25.00	5,622.25
L-serine	384.19	20.00	7,683.80
L-tyrosine	183.47	40.00	7,338.80
Unassessed amino acids	534.95	25.00	13,373.75
Fatty Acid Total	51.71		1,540.99
Lauric acid	3.64	20.00	72.80
Myristic acid	2.05	50.00	102.50
Palmitic acid	25.51	50.00	1,275.50
Stearic acid	4.73	5.00	23.65
cis-oleic acid	12.69	5.00	63.45
cis-linoleic acid	3.09	1.00	3.09
Other Content	34,973.48		89,288.33
Other lipids	616.49	1.00	616.49
Carbohydrates	25,632.70	2.20	56,391.94
Minerals (ash)	8,724.30	3.70	32,279.91
Water	11,439.87	0	0
Total	**52,000.00**		**222,924.60**
Economic Value of a Kilogram of Seaweed Biomass Waste (USD)			4.29

The implementation of biorefinery on seaweed biomass waste would be preferable if it also applies mild conditions, fewer energy inputs, and low or zero pollutants generation. Water and solvent recycling, enzyme application (especially immobilized enzyme), and green extraction methods are some operational options that could be implemented to achieve those purposes [9, 29, 34, 47, 48]. Moreover, the seaweed biomass waste biorefinery should be proceeded by laboratory works and pilot-scale test, complemented by techno-economic analysis (TEA), and life-cycle assessment (LCA) before realization on an industrial scale, to ensure that the biorefinery process generates economic profits and no harmful environmental impacts [3, 45, 49].

DOI: 10.5614/3bio.2022.4.1.6

4. Conclusion

This case study has shown that seaweed *Gracilaria* sp. biomass waste from the representative seaweed production unit in Karawang, Indonesia (generated at approximately 52 metric tons/year) still contains bioactive components with potential gross revenue of ± USD 222,924.6/year or an economic value of USD 4.29/kg of seaweed biomass waste. The biorefinery approach could be applied to generate high-value products sustainably. Hopefully, this study could be a reference for accelerating the development of local seaweed biomass-based bioindustry with high economic competitiveness and environmentally friendly.

Acknowledgments

The authors would like to thank Mr. Usup Supriatna, the head of Koperasi Mina Agar Makmur, who has provided information on the dried seaweed pressing process at his cooperative and allowed the authors to analyze the seaweed biomass waste generated from his production unit. This study received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors. The authors declare that there is no conflict of interest.

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DOI: 10.5614/3bio.2022.4.1.6

e-ISSN: 2655-8777 http://journals.itb.ac.id/index.php/3bio

Correlation of Microclimate of West Java on Caffeine and Chlorogenic acid in *Coffea canephora* var. *robusta*

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Received: 2022-02-24
Accepted for publication: 2022-04-13

Abstract

Caffeine and chlorogenic acid (CGA) are two compounds that play a role in determining the quality of coffee. The amount of the two compounds may vary depending on the environment where they are grown. This study aimed to determine the correlation between the local microclimatic condition and the concentration of caffeine and CGA in green and roasted beans of Robusta coffee from six different cultivation areas in West Java, Indonesia (i.e., Ciamis, Tasikmalaya, Sumedang, Kuningan, Cianjur, and Bogor). Samples of green beans and roasted beans were extracted with 70% methanol for caffeine analysis and ethyl acetate for CGA analysis. Caffeine and CGA were analyzed by UV-HPLC using a C18 shimpack gist shimadzu column, with an isocratic elution of methanol:water (1:1) at a 1 mL/min flow rate. Detection was performed at λ272 nm and λ324 nm for caffeine and chlorogenic acid, respectively. Principal component analysis (PCA) was used to evaluate the correlation between microclimate with caffeine and chlorogenic acid. Results indicated that the concentration of caffeine ranged from 7.67 to 16.52% and 10.79 to 15.56% in the green and roasted bean coffee, respectively. The concentration of CGA ranged from 0.74 to 3.03% and 0.25 to 0.77% in the green and roasted bean coffee, respectively. Based on PCA analysis, the most influential microclimate on the caffeine concentration were the humidity, temperature, and altitude, with the total variance of PC1 and PC2 of 76.3%. However, there was no positive correlation between the measured microclimate and the CGA concentration. In conclusion, Robusta coffee's caffeine content is positively affected by the microclimatic condition (i.e., humidity, temperature, and altitude).

Keywords: caffeine, chlorogenic acid, Robusta, microclimate, Java coffee

1. Introduction

Indonesia is known as the third largest coffee producer globally after Brazil and Vietnam [1,2,3,4]. The region of Coffee production exists in almost all parts of Indonesia, including West Java. Based on the Regional Rule of the Province of West Java concerning Guidelines for the Implementation of Cultivation, coffee is a commodity that has an essential role in building the economy of the society of West Java [5,6,7].

Coffee in West Java consists of two types, i.e., Arabica and Robusta coffee. Arabica coffee is more suitable for being grown at high altitude, while Robusta coffee can be grown at lower altitudes. The average productivity of Robusta coffee is 784 kg/Ha. The area cultivation of the Robusta coffee in West Java is around 15.750 Ha [35]. West Java Robusta coffee has been marketed to local and international markets. Robusta coffee from Ciamis, Sumedang, Tasikmalaya, and Bogor has been locally marketed to other provinces in Indonesia. In contrast, Robusta coffee from Cianjur and Kuningan has been internationally marketed to other countries such as Saudi Arabia, Morocco, Singapore, Turkey, and France.

In general, Robusta coffee from various regions in West Java has different flavors, such as sweetness and acidity [30]. Many factors influence the differences in Robusta coffee flavors, such as natural and artificial factors [35]. Natural factors include geographical conditions, genetics or varieties/species, microclimates conditions, and fruit maturity. Artificial factors such as post-harvest processing include preparation, grinding, drying, washing, and roasting temperature [8,9,26,35].

The demand for West Java Robusta coffee generally is related to the coffee quality, especially the taste of coffee. The taste of coffee is influenced by the composition of the metabolites in the beans [13,14]. Some coffee beans'

DOI: 10.5614/3bio.2022.4.1.7

metabolites, e.g. sugar, caffeine, theobromine, theophylline, trigonelline, and chlorogenic acid, can significantly affect the taste [27,28,29]. Two of them were caffeine and chlorogenic acid [15,16,17]. Caffeine plays a role in determining the bitter taste of coffee, and chlorogenic acid in coffee's sour taste [18,19,20,21,22,36]. An increase in caffeine concentration in the coffee beans indicates a good quality of coffee. In contrast, the rise of CGA concentration in the coffee beans indicates poor quality [8].

According to previous studies, environmental factors such as altitude and microclimate (i.e., temperature, humidity and rainfall) correlate with caffeine and CGA concentration [10,11,12,17]. However, the concentration of caffeine and CGA negatively correlates with altitude [37]. In addition, lower temperatures and low intensity delay the coffee maturity, thus, inhibiting some metabolites' accumulation in coffee beans [11,23]. Moreover, no significant effect was found between CGA concentration and rainfall, but the temperature positively correlates with the caffeine concentration and CGA [23,24].

Research on the analysis of caffeine and CGA concentrations in coffee has been done previously by Jeszka et al. [34], Awwad et al. [36], Girma et al. [37], and Hagos et al. [38]. None of those researches analyzed caffeine and CGA concentration of Robusta coffee beans from West Java. Therefore, the objective of this study was to analyze the caffeine and CGA of Robusta coffee from West Java and to determine the correlation between microclimates with the concentrations of caffeine and CGA. Cultivation area of Robusta coffee in West Java selected which has area more than 1000 Ha, including the areas of Bogor, Ciamis, Cianjur, Kuningan, Sumedang, and Tasikmalaya.

In this study, High-Performance Liquid Chromatography (HPLC) was used to analyze caffeine and CGA as HPLC is an effective method for analyzing caffeine and chlorogenic acids in the coffee bean and roasted bean [32,33]. The concentration of caffeine and CGA were correlated to the microclimate (temperature, rainfall, and humidity) and altitude using a multivariate statistical. The Principal Component Analysis (PCA) in this study was used to determine the correlation between each variable and the concentration of caffeine and CGA.

2. Methodology

The sample of six green and six roasted coffee beans were obtained from six different areas in West Java (Bogor, Ciamis, Cianjur, Kuningan, Sumedang, and Tasikmalaya). Robusta Coffee from those cultivation areas was subjected to caffeine and CGA analysis using HPLC. The concentration of caffeine and CGA was correlated to microclimates (temperature, rainfall, and humidity) and the altitude of where the coffee originated. Multivariate statistical analysis was used to interpret the data through Principal Component Analysis (PCA).

2.1. Samples preparation

Green beans Robusta coffee were obtained from smallholder farmers from six different cultivation areas in West Java (i.e., Ciamis, Tasikmalaya, Sumedang, Kuningan, Cianjur, and Bogor). Roasted samples of Robusta coffee were prepared using a coffee roaster machine. The samples of coffee beans were ground using a coffee grinder, Cyprus grinder GR0063. The ground green bean coffee was put quickly into a 30 ml dark glass bottle to avoid the evaporation of volatile compounds. Bottles containing samples of roasted and green bean coffee were stored at room temperature before further processing.

2.2. Extraction

0.25 g of green beans and roasted beans from each location were separately extracted with 30 mL of methanol/water (70:30 v/v) for caffeine analysis and ethyl acetate for chlorogenic acid analysis. The extracts were sonicated at 65°C for 10 minutes and centrifuged at 1372 xg for 3 minutes. This procedure was repeated three times, and the supernatant was collected at the end of each centrifugation cycle. The supernatant from each sample was evaporated using a vacuum evaporator to obtain a crude extract. Then, it was filtered using a polytetrafluoroethylene (PTFE) syringe filter with a size of 0.22µm, according to the method of Ceylan et al. [3].

2.3. Caffeine and CGA analysis

The analysis of caffeine and chlorogenic acid in the green and roasted bean samples was conducted by Shimadzu Prominence type 20A HPLC. Twenty µL of each sample was applied to the HPLC using Shimpack C18 gist shimadzu column (5 µm particles, 4.6 mm internal diameter, and 25 cm length). The analysis was carried out at 27 °C using isocratic elution of methanol:water (50:50) with a flow rate of 1 mL/min. A UV detector was used at λ272 nm and λ324 nm to detect the caffeine and chlorogenic acid peaks, respectively.

Standard solutions of caffeine ad CGA were prepared in separate flask by appropriate dilution of 0.1 mg/mL (100ppm) stock solutions with the same solvent to contain 0.5,0.25,0.125, and 0. 0625 mg/ml final concentration. Each standard solution was injected into HPLC in triplicate. Calibration curves were obtained by plotting the peak areas of each standard solution versus the concentration of the injected standard solution.

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2.4. Microclimate

Data of temperature, humidity, and rainfall were average per year obtained from the data center of the National Statistics Agency on the website (https://www.bps.go.id/), and data on altitude was obtained from Google Earth 2021.

2.5. Statistical analysis

The data were processed by Minitab version 17 followed by principal component analysis (PCA) using Minitab version 17 to evaluate the correlation between microclimate with caffeine and chlorogenic acid.

3. Results and discussion

3.1. Caffeine and CGA concentration

Results showed that the caffeine concentration in green beans and roasted beans from six coffee plantation areas ranged from 7.67–16.52% in green beans and 10.79-15.56% in roasted beans, respectively (Table 1.). The lowest caffeine concentration was found in coffee samples from Ciamis, both for green bean coffee (7.67%) and roasted bean coffee (10.79%). The highest caffeine concentration was found in the green bean coffee sample from Cianjur (16.52%) and the roasted bean coffee sample from Bogor (15.46%). In this study, the Robusta coffee has a high concentration of caffeine. This finding was in line with the study by Cheng et al. [17] and Sunarhanum et al. [8], who mentioned that the concentration of caffeine in Robusta coffee was higher than in Arabica coffee. The study also showed that the concentration of caffeine in coffee beans before and after the roasting process was not much different. This result was correlated with a previous study by Cheng et al.[17] and Jeszka et al. [34] and supported by the theory that caffeine has thermostable characteristics [17].

Chlorogenic acid concentration ranged from 0.74-3.03% in green bean coffee and 0.25-0.77% in roasted bean coffee, respectively (Table 1.). The lowest concentration of chlorogenic acid was found in the green bean coffee sample from Cianjur (0.74%) and the roasted bean coffee sample from Tasikmalaya (0.25%). The highest concentration of chlorogenic acid was found in the green bean coffee sample from Sumedang (3.03%) and in the roasted bean coffee sample from Kuningan (0.77%). The study also showed that the concentration of chlorogenic acid in coffee beans before and after the roasting process is not much different. This result agrees with the study of Cheng et al. [17] and is supported by the theory that chlorogenic acid has thermostable characteristics [17].

Caffeine concentration increased after the roasting process, while CGA concentration decreased (Table 1.). The change in caffeine and chlorogenic acid concentration after roasting was affected by high temperatures during the roasting process, as reported by Sunarharum et al. [8]. The temperature and duration of the roasting process affect caffeine concentration and a drop of chlorogenic acid [8]. The roasting process plays a role in determining the taste and quality of brew coffee. Several volatile and nonvolatile compounds were formed during the roasting process and contribute significantly to the sensory characteristics of coffee drinks. Certain compounds' intensity varies depending on the temperature of roasting processed, particularly caffeine and chlorogenic acid. During the roasting process, chlorogenic acid was degraded into lactone, which caused the quantity of CGA to decrease [25]. On the other hand, caffeine acid releases caffeine during the roasting process, increasing the total caffeine concentration [31].

3.2. Correlation of caffeine and CGA with altitude and microclimates

The PCA analysis reveals that the caffeine and chlorogenic acid concentrations were affected by different microclimatic conditions. Caffeine concentration was positively correlated with humidity, altitude, and temperature. On the other hand, caffeine does not correlate with rainfall. The chlorogenic acid concentration was not correlated with the microclimate. PCA results showed that the most influential microclimatic variable to caffeine concentration was the humidity, altitude, and temperature, with a variance value of 47.8% (PC1) and 28.5% (PC2), or 76.3% in total (Figure 1).

The microclimate variables, altitude, temperature, rainfall, and humidity were not correlated with the concentration of CGA, with a variance of 37.2% (PC1) and 34.1 (PC2) or a total of 71.3% (Figure 2.). Caffeine concentration was positively correlated with humidity, altitude, and temperature, while CGA concentrations were not correlated with microclimatic variables (humidity, temperature, and rainfall) and altitude. Altitude was negatively correlated with CGA concentration [17,37,38]. Indeed, the coffee from Cianjur with the highest altitude (713 masl) showed the lowest concentration of CGA (0.74%). A similar result was also reported by Girma et al. [37] and Hagos et al. [38].

According to Odeny et al. [10], environmental factors influence caffeine accumulation [10]. For example, lower temperatures, high altitude, and high humidity delay coffee maturity for about one month and cause the biochemical composition to variate significantly at the stage of bean development. Delay coffee maturity causes the accumulation of caffeine and CGA [23,11]. However, no relationship was found between caffeine and CGA concentration and rainfall (Figure 2.). A similar result was also reported in another study by Joet et al. [23].

DOI: 10.5614/3bio.2022.4.1.7

Table 1. The concentration of caffeine and chlorogenic acid in the green bean and roasted bean Robusta coffee samples from six areas of West Java.

Sample coffee	Region	Concentration (%) (v/v)		water content (%)	Microclimates*			Altitude (masl**)
		CGA (%)	Caffeine (%)		Temp. (ºC)	Humidity (%)	Rainfall (mm/year)	
Green Bean	Bogor	1,86	11,69	8,13	25,5	83	3930	700
	Ciamis	3,0	7,67	4,28	25	74,8	1309	607
	Kuningan	3,03	11,07	4,99	27	74	1682	657,5
	Sumedang	2,28	12,13	4,85	24,7	69	2570	551
	Tasikmalaya	1,77	10,14	3,31	25,7	67	2750	398,5
	Cianjur	0,74	16,52	4,04	26,5	88	1250	713
Roasted Bean	Bogor	0,27	15,46	1,81	25,5	83	3930	700
	Ciamis	0,32	10,79	0,92	25	74,8	1309	607
	Kuningan	0,77	12,62	3,10	27	74	1682	657,5
	Sumedang	0,32	11,09	1,57	24,7	69	2570	551
	Tasikmalaya	0,25	11,43	1,10	25,7	67	2750	398,5
	Cianjur	0,49	10,82	3,53	26,5	88	1250	713

*Data of microclimates were obtained from the data center of the National Statistics Agency on the website (https://www.bps.go.id/), while data on altitude was obtained from google earth 2021.

**Above the sea level

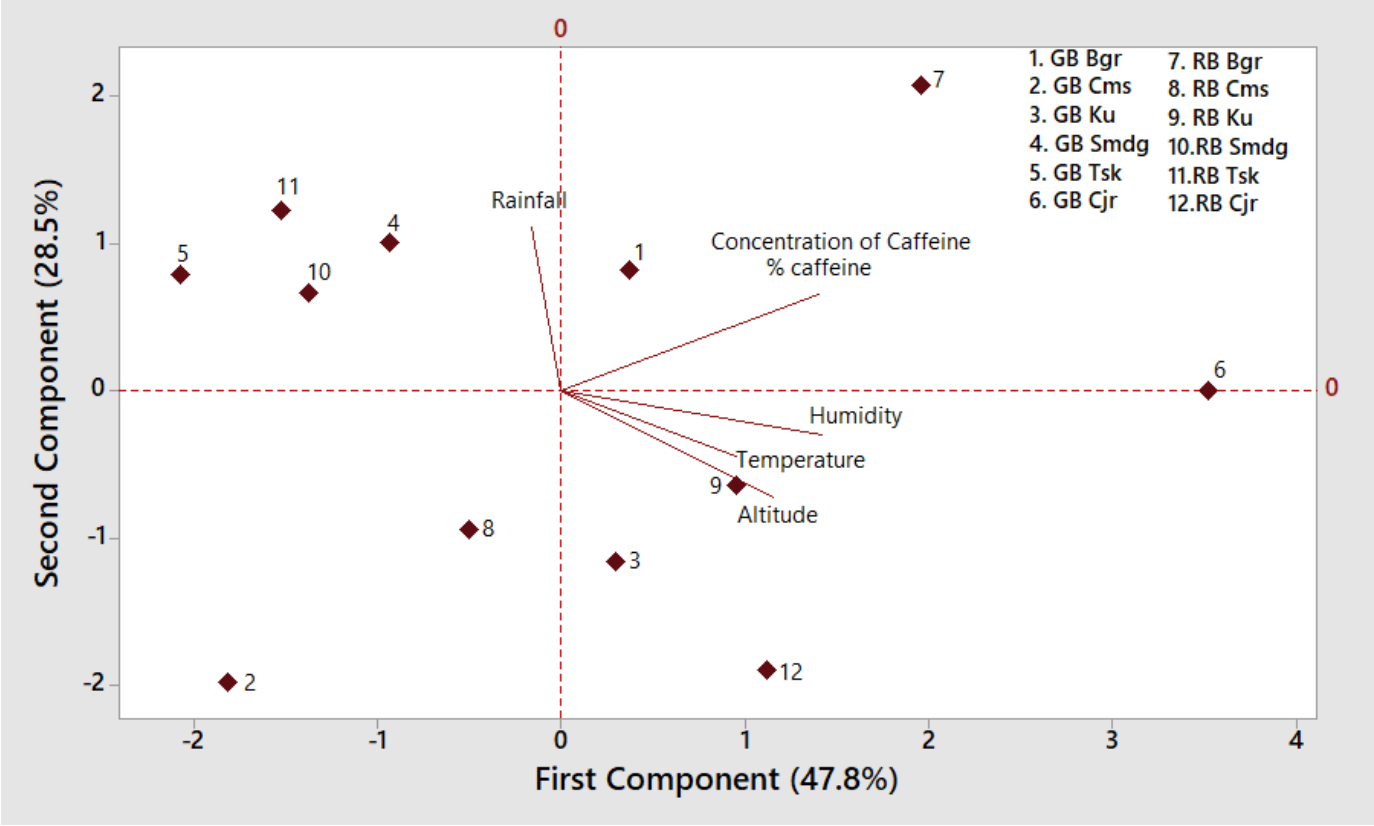

Figure 1. PCA biplot of microclimatic (altitude, humidity, rainfall, temperature) on caffeine concentration (GB = green beans, RB = roasted beans, Bgr = Bogor coffee samples, Cms = Ciamwas coffee samples, Ku = Kuningan coffee samples, Smdg = sample of Sumedang coffee, Tsk = sample of Tasik coffee and Cjr = sample of Cianjur coffee).

DOI: 10.5614/3bio.2022.4.1.7

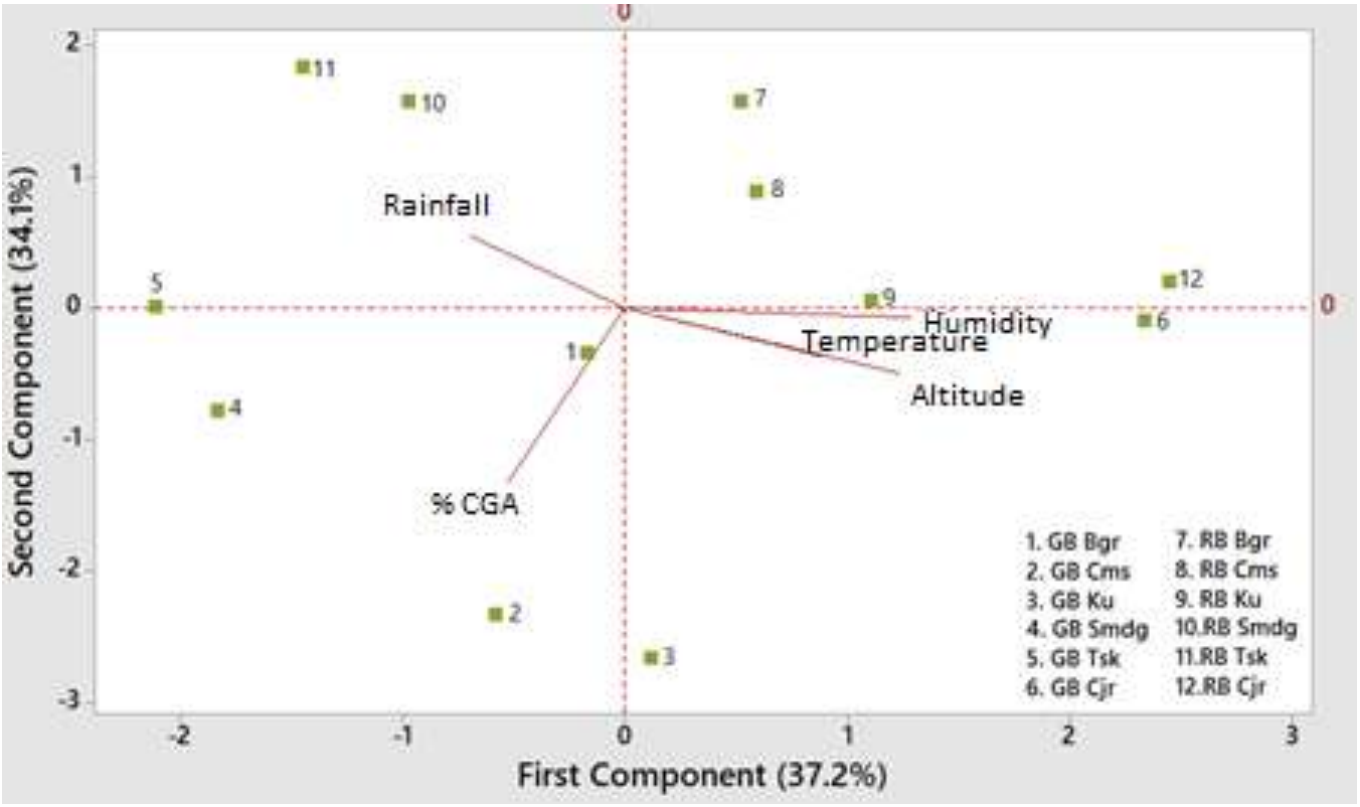

Figure 2. PCA biplot of microclimatic (altitude, humidity, rainfall, temperature) on CGA concentration (GB = green beans, RB = roasted beans, Bgr = Bogor coffee samples, Cms = Ciamwas coffee samples, Ku = Kuningan coffee samples, Smdg = sample of Sumedang coffee, Tsk = sample of Tasik coffee and Cjr = sample of Cianjur coffee).

4. Conclusion

There is a positive correlation between humidity, temperature, altitude, and caffeine concentration. However, no correlation between the microclimate with the concentration of CGA.

Acknowledgments

The authors acknowledge financial grants provided by P2MI Institut Teknologi Bandung, Indonesia to Erly Marwani. We also acknowledge to Lembaga Pengelola Dana Pendidikan (LPDP) who gave Master degree program scholarship to Suci Awaliyah.

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DOI: 10.5614/3bio.2022.4.1.7

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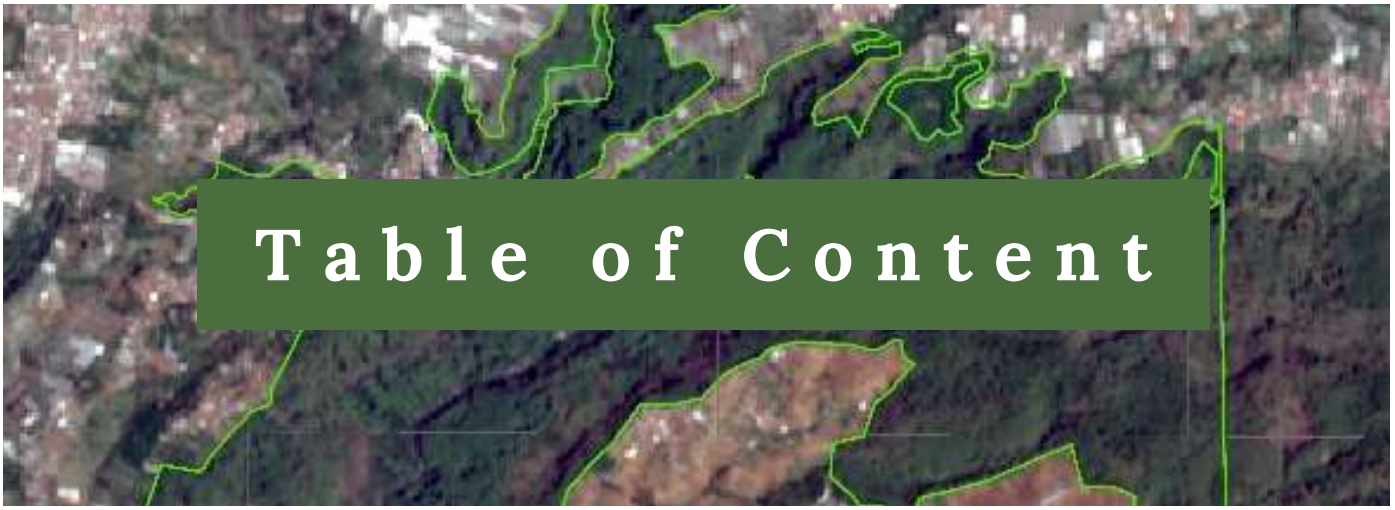

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