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Business Model Canvas (BMC) Approach for Ecotourism Development Based on Islamic Boarding School Community(Case: Cipeujeuh Valley, Darul Arqam Muhammadiyah Islamic Boarding School)

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Abstract

This study investigates the potential for ecotourism in Cipeujeuh Valley, which is situated near the Darul Arqam Muhammadiyah Islamic boarding school in Garut Regency. The research examines the factors that drive tourists to visit the area, the level of community engagement, and the development of a community-based ecotourism business model. The data was gathered using descriptive statistics, the Ecotourism Opportunity Spectrum (ECOS) framework, and rapid rural appraisal techniques. The results indicate that Cipeujeuh Valley has intermediate ecotourism spectrum, with push factors such as sports and adventure, recreation and relaxation, social status, and quality time, and pull factors including nature and the local community, and enjoying facilities. The community is generally receptive to ecotourism development, with 61.3% expressing interest and 38.7% not. The study suggests that educational institutions, communities, organizations, and families can be targeted through a business model canvas that offers unique environments, sustainable resource use, community empowerment, educational tourism, and economic development. Key resources include natural attractions, religious and local wisdom activities, infrastructure, and a skilled workforce, while key activities involve nature-based and community-based tourism experiences.

Keywords: *Business Model Canvas, Darul Arqam Islamic Boarding School, Ecotourism*

1. Introduction

The COVID-19 pandemic has had a profound and far-reaching impact on Indonesia's economy, particularly in the tourism sector, as well as on the health sector. The tourism industry plays a vital role in generating state revenue and stimulating economic growth. According to the Ministry of Tourism and Creative Economy, the pandemic resulted in a significant decrease in income for the tourism sector, amounting to a total of Rp. 20.7 billion. The pandemic's visible impact on the tourism industry in Indonesia is evident in the significant decline in the number of global tourist arrivals, which plummeted from 847 million to 1,139 million people, resulting in a drop of 58-78%. Furthermore, the COVID-19 pandemic has had a direct and negative impact on workers in the Indonesian tourism sector.

As the global situation continues to improve, the tourism industry is gradually returning to normalcy, with ecotourism becoming increasingly popular. It is anticipated that communities will increasingly prefer eco-based tourism and outdoor activities when selecting travel destinations. Among the institutions that can significantly contribute to the economy are Islamic boarding schools. Although these institutions are often misconstrued as focusing solely on religious education, they have the potential to serve as significant economic drivers closely intertwined with the local community. Despite being one of the oldest educational institutions in Indonesia, Islamic boarding schools can play a vital role in promoting sustainable tourism and enhancing the local economy.

The potential for economic development through tourism exists at Darul Arqam Muhammadiyah Islamic Boarding School in the Garut Region, one of the Islamic boarding schools inWest Java. In both developed and developing countries, tourism has been proven to be a successful driver of rural economies [1]. The underutilized Cipeujeuh Valley presents an opportunity to develop its ecotourism potential. By harnessing this potential, the strategic role of Islamic boarding schools in supporting the regional development of

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Garut Regency can be realized. This aligns with the Garut Regency Regional Regulation No. 2 of 2019, which focuses on the 2019-2025 Regional Tourism Development Master Plan. Darul Arqam Muhammadiyah Islamic Boarding School in the Garut Region, located in Cilawu District, is included in the Regency Tourism Development Area (KPPK), which covers natural, cultural, and artificial tourism development.

To fully study all aspects of the development, a business model is required to facilitate planning and governance for the ecotourism potential in Cipeujeuh Valley, with Islamic boarding schools at its core. The Business Model Canvas (BMC) is a model that describes how a business is created, delivered, and captured, enabling business planners to effectively depict and modify business models, generating new strategic alternatives that can be readily comprehended by diverse groups [2]. The purpose of this research is to analyze the ecotourism spectrum of Cipeujeuh Valley based on the Islamic boarding school community, the push and pull factors motivating tourist visits, the response and participation level of the boarding school community, and the business model planning for the development of community- based ecotourism in Cipeujeuh Valley. The business model canvas is a fundamental tool in this study, and it is expected to be implemented for the economic development of Islamic boarding schools, thereby aiding the government in

2. Methodology

This study was carried out from November 2022 to June 2023 at the Darul Arqam Muhammadiyah Islamic Boarding School in the Garut Region, which is situated at Jl. Raya Garut-Tasikmalaya No. 36, RT 03/RW 02, Ngamplangsari Village, Cilawu District, Garut Regency, West Java Province. The research focused on the Cipeujeuh Valley, which is located in the region behind the Islamic boarding school (see Figure 1). The study employed various data collection methods, such as observation, interviews, questionnaires, and literature study. The sampling method used was purposive sampling, selecting individuals who were considered knowledgeable in providing input on the business model canvas design. A total of 93 respondents participated in the study, including Cipeujeuh Valley managers, tourists who had visited the area, and individuals residing at the Darul Arqam Islamic boarding school.

Data analysis within this study comprised descriptive analysis, identification of ecotourism potential, examination of visitor motivation, and evaluation of community engagement within the Islamic boarding school. The identification of ecotourism potential was facilitated through the utilization of the Ecotourism Opportunity Spectrum (ECOS) framework, which entailed calculating the Recreational Zone Index (RZI) value to determine a suitable ecotourism spectrum. This approach provided a practical means for destination development, as indicated by the specified reference [3]. The formula employed in this process is as follows:

$$RZI = \Sigma\left(\frac{Ni}{Nmax}\right) \times 100\%$$

With:

Ni = I-th variable value

N1 = Access components

N2 = Related resources components

N3 =Attraction components

N4 = Infrastructure components

N5 = Components of ability and knowledge

N6 = Components of social interaction

N7 = Visitor impact components

Nmax = Maximum value of all categories

The outcomes of the calculations can be categorized into the ecotourism spectrum in accordance with Table 1.

An examination of visitor motivation was conducted through a push and pull factor analysis, which facilitated comprehension of the factors and requirements that tourists consider when traveling [4]. The engagement of the Islamic boarding school community was assessed using rapid rural appraisal methods, which enabled a swift evaluation of the community's circumstances and the development of plans and actions to enhance their standard of living [5]. Based on these assessments, the subsequent step involved the development of a business model utilizing the Business Model Canvas [2] to effectively map out a suitable model for the advancement of the Cipeujeuh Valley. The model is employed to elucidate the business logic that has been constructed and developed by encompassing four crucial areas: customer, offering, infrastructure, and financial feasibility. These nine components can serve as a validation form of the development potential of a business idea. The following are the specifics from Business Model Canvas: customer segment, value proposition, channel, customer relationship, revenue streams, key resources, key activities, key partners, and cost structures.

Table 1. RZI Assessment

No	Index	Ecotourism Spectrum
1	>67%	Eco Specialist (ES)
2	66%-34%	Intermediate (IM)
3	0%-33%	Eco Generalist (EG)

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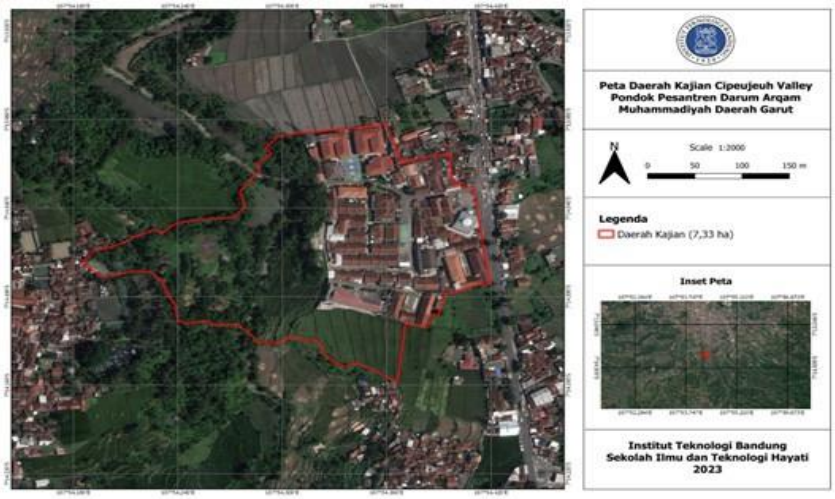

Figure **1**. Research Study Area

3. Results and Discussion

3.1 Ecotourism Potential

Analyses of the potential for ecotourism in the Cipeujeuh Valley, utilizing Islamic boarding schools, were conducted using the ECOS framework. The analysis entailed calculating a spectrum assessment with the RZI formula [6], which incorporated seven parameters. The value of each parameter was employed in the calculation. The eco-specialist spectrum was essentially a value of "3" the intermediate spectrum was assigned a value of "2" and the eco-generalist spectrum was given a value of "1".

According to the information in Table 2, the RZI value can be determined using the following formula:

The RZI value of 52.38% signifies that the ecotourism spectrum of the Cipeujeuh Valley falls within the intermediate range, as calculated below.

$$RZI = \Sigma\left(\frac{Ni}{Nmax}\right) \times 100\%$$

$$RZI = \Sigma\left(\frac{11}{21}\right) \times 100\% = 52.38\%$$

The ecotourism spectrum that falls between hard ecotourism and soft ecotourism is known as the intermediate ecotourism spectrum [3]. Hard ecotourism attractions include intense overnight trips to national parks, while examples of soft ecotourism include forest climbing with short track trails and health-oriented spas and catering services. The development of the intermediate ecotourism spectrum can be observed in the formation of visitor patterns that can be used to segment customers. This can lead to an increase in the number of visits and changes in tourist expectations, as well as an increased awareness of the environment in the tourist destination. Travelers who participate in ecotourism with an intermediate spectrum typically visit in small groups, using transportation commonly used for tourism, and utilize the facilities and services provided by tourism management.

Table 2. ECOS Component Value

No	Component	Spectrum	Value
1	Access	Eco-Generalist	1
2	Activity	Eco-Generalist	1
3	Attractions offered	Intermediate	2
4	Infrastructure	Intermediate	2
5	Social Interaction	Eco-Generalist	1
6	Manager's Knowledge and Skill Level	Intermediate	2
7	Impact of Visitors' Acceptance on The Nature	Intermediate	2
Total			11

The diverse range of eco-tourism attractions available at Cipeujeuh Valley and Darul Arqam Islamic Boarding School provide visitors with a variety of activities that showcase the natural surroundings. At Cipeujeuh Valley, educational experiences such as hands-on learning about the environment and outdoor activities that teach children about nature are available. In addition, adventure sports like tracking, archery, river tubing, and flying fox are offered

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to promote physical and spiritual well-being. Visitors can also learn about agriculture and partake in activities such as vegetable and fruit picking and enjoying the culinary harvests. Furthermore, camping activities provide an exciting outdoor living experience. All these activities are aimed at fostering environmental awareness, promoting health, and offering a fun and educational recreational experience. A visual representation of these activities can be found in Figure 2.

Figure **2.** Ecotourism Attraction. (a) River Tubing, (b) Agrotourism, (c) Camping

3.2 Interpretation of Push Factors and Pull Factors

Following extensive testing to evaluate push and pull factors that motivate tourists, the study has produced results that identify and describe the factors, along with their respective percentages of overall contribution.

3.2.1 Push Factors

The four factors comprising the tested push variables were identified through a thorough analysis. It is essential to provide an interpretation and appropriate naming for these factors. The factor loading values play a crucial role in determining the influence of each variable on other variables. Hence, variables with higher factor loading values are expected to have a more significant impact on representing other factors. The factors were named based on the distinct characteristics of each item within the factor, which is clearly demonstrated in Table 3.

Table 3 in the text provides an interpretation and naming of each factor that influences tourist motivation for eco-tourism in the Cipeujeuh Valley. The factors are named based on the variables they contain. The first factor, named "sports and adventure," is related to the psychological happiness that comes from engaging in sports and adventure activities. This happiness is derived from fulfilling basic psychological needs such as autonomy, competence, and social connectedness, as well as from a connection with nature [7]. The second factor, named "recreation and relaxation," has a positive correlation with nature-based recreation and mental health, leading to improved mood, cognition, recovery, and well-being, and potentially reducing anxiety and depression [8]. The third factor, named "social status," is related to individuals using tourism travel to display and affirm their social status to friends, relatives, and colleagues [9]. Lastly, the fourth factor, named "quality time," involves spending quality time with family, which can strengthen family bonds, alleviate stress and tension, foster better relationships, and benefit family members [10].

3.2.2 Pull Factors

The Pull variables were categorized into two factors, and it is essential to provide an interpretation and naming of these factors. The variables that exhibit higher factor loading values are considered to be more influential in representing the other factors. The factors were named based on the characteristics of each member in the factor, as illustrated in Table 4.

Table 4 provides a comprehensive interpretation and categorization of the factors influencing tourist motivation for eco-tourism in the Cipeujeuh Valley. The factors are named based on the variables contained within each category. The first factor, "nature and local community," encompasses

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Table 3. Interpretation of Push Factors

Factor	Variable	Factor Loading	Description
Sports and Adventure (22.58%)	X18	881	Testing Adrenaline
	X16	783	Desire to do challenging activities
	X15	779	Exercise physical strength
Recreation and Relaxation (22.42%)	X04	854	Get rid of stress
	X05	836	Take advantage of vacation time
	X03	825	Break away from fullness
	X01	736	Looking for a new experience
Social Status (15.20%)	X07	847	Looking for a luxurious and exclusive place
	X09	817	Show social class and prestige
	X11	746	Meet new people
Quality Time	X10	794	Spend time with your partner/family/friend

the natural beauty and charm of the village, the pristine environment, fresh air, appealing photography spots, and a peaceful atmosphere that attracts tourists to visit. Additionally, an interest in the local community contributes to achieving a balance between the environment, economy, society, and culture in sustainable tourism development. The interaction between local communities and tourists serves as a means for them to connect and engage socially, as stated in reference [11]. The second factor, "enjoying facilities," considers the availability of accommodations and transportation, which are key attractions for visitors. The appeal of these amenities, both domestically and internationally, determines their value as assets in the tourism industry, as noted in reference [12].

3.3 Islamic Boarding School Community Participation

Following a Rapid Rural Appraisal, the findings indicated that 61.3% of the community expressed their readiness to participate, while 38.7% indicated their unwillingness to be involved in the development of Cipeujeuh Valley ecotourism.

The diverse range of individuals who have expressed interest in contributing to the development of Cipeujeuh Valley encompass a variety of roles, including the upkeep and management of agrotourism gardens, serving as facilitators for educational programs designed for children, acting as guides for tourists on excursions, providing instruction on Islamic history and interpreting religious texts such as the Quran and hadith, as well as selling pre-packaged products.

The abilities of the community members contribute

Table 4. Interpretation of Pull Factors

Factor	Variable	Factor Loading	Description
Nature and Local Community (38.04%)	X20	814	The atmosphere and the air
	X25	805	Local culture
	X19	797	Interesting environment and nature
	X26	782	Local community activities
	X23	709	Natural produce produced
	X27	708	Local community hospitality
Enjoying Facilities (29.53%)	X29	892	Enjoy accommodation facilities
	X28	841	Visiting artificial tourist rides and games
	X30	766	Enjoying other facilities

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significantly to the development of Cipeujeuh Valley ecotourism. These skills encompass foreign language proficiency, artistic talents, culinary expertise, sports skills, and souvenir making. The knowledge of foreign languages, particularly English and Arabic, can be utilized for language-related activities such as storytelling, language lectures, poetry readings, and writing stories, among other language- related activities. The artistic abilities of the community members encompass performances in karawitan, angklung, traditional thread art, and various other artistic activities. Additionally, the preparation of special local dishes can serve as an activity to introduce culinary specialties and showcase the local culture. The community can also guide workshops on Sundanese cuisine. Furthermore, the sports skills of the community members can be utilized both in Cipeujeuh Valley and at Darul Arqam Islamic boarding school, with individuals serving as guides and coaches in sports such as basketball, volleyball, jogging, gymnastics, archery, traditional thread games, and other sports.

The participation of the Islamic boarding school community in the management of ecotourism can offer chances for a variety of activities, serve as additional sources of income, preserve cultural heritage, and contribute to the conservation efforts of the ecotourism area [13].

3.4 Business Model Canvas

The objective of creating the Business Model Canvas (BMC) is to establish a strategic basis for Darul Arqam Islamic Boarding School's participation in the Cipeujeuh Valley ecotourism development business plan, which is specifically designed for the Islamic boarding school community.

3.4.1 Customer Segment

The offerings in Cipeujeuh Valley's ecotourism sector, which are focused on the Islamic boarding school community, encompass a diverse array of activities. These activities include educational experiences, agrotourism, sports and adventure, recreation and relaxation, as well as opportunities for social status enhancement, quality time spent with loved ones, and engagement with activities inspired by the natural surroundings and local community. The facilities already exist and are utilized in order to cater to a wide range of customers, including preschool to college students, organizations, communities, and families, who all fall within the customer segmentation of Cipeujeuh Valley.

3.4.2 Value Proposition

The factors that influence tourists' decisions to visit Cipeujeuh Valley and are used to determine their interest in its attractions are presented in Table 5. These values serve as a crucial element in the decision-making process.

Factors that influence tourists' decisions to visit Cipeujeuh Valley and their interest in its attractions are presented in Table 5. These factors serve as a vital element in the decision-making process.

The Cipeujeuh Valley's value propositions are numerous and diverse. The Islamic Boarding School environment, which is steeped in religious significance and represents the Islamic community's way of life, is a highly attractive destination for tourists. Additionally, the valley utilizes natural resources as tourist attractions, which helps to conserve the environment while also generating economic benefits. By empowering the Islamic Boarding School community to participate in managing and operating tourism activities, they are provided with additional sources of income. Edu-tourism offers visitors the opportunity to enjoy learning and enrich their experiences, while eco-tourism fosters environmental education and a love for nature, contributing to the Islamic Boarding School's economy.

3.4.3 Channel

The different channels that Cipeujeuh Valley can utilize to promote its tourist products to potential visitors, thus minimizing marketing costs, are presented in Table 6.

The Cipeujeuh Valley's channels have been categorized into three types: owned, partner, and customer channels. Owned channels include direct web sales via marketing on the website and social media platforms like Instagram, Facebook, and Twitter. Business-owned channels involve distributing printed tourism maps in clinics, laundries, catering services, and cooperatives. Partner channels are indirect and involve collaboration with various stakeholders, including the pesantren community (students, mentors, teachers, and staff), local small and medium-sized enterprises (SMEs), alumni associations, parents of students, the Garut Regency government, and tourism agencies. Lastly, customer channels rely on word-of-mouth from visitors.

3.4.4 Customer Relationship

To build a strong relationship with consumers, Cipeujeuh Valley's management must undergo three distinct phases. The initial phase entails the acquisition process, during which digital promotion is executed via various partnerships, such as collaborations with Islamic boarding schools' community, alumni networks, and the local government of Garut Regency. Complementary to the campaign, pertinent information about tourism products will be disseminated through virtual videos and website pages. Patrons can easily

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Table 5. Cipeujeuh Valley's Value Proposition

Cipeujeuh Valley Value Proposition	
Islamic boarding school Environment	The Islamic boarding school environment is an interesting environment to visit because it is closely related to religion and is a miniature of the life of the Islamic community
Natural Resource Utilization	The use of natural resources as attractions offered can be a solution to keep environmental conservation running along with economic income
Empowering the Community of Islamic boarding school	Helping the Islamic boarding school community to be involved in the management and travel of tourism activities so that they can then become an additional source of income for their economy
Edu Tourism	The provision of tourism-based education makes learning fun and adds interesting experiences
Economic Development of Islamic boarding school	Ecotourism is one of the major sources of income for Islamic boarding school that is close to education and love for the environment

register and reserve travel packages, both online and offline.

The subsequent stage is the retain phase, during which Cipeujeuh Valley management must create a positive and lasting first impression to ensure that tourists have a delightful experience from the very beginning. This involves presenting them with welcome beverages and snacks, such as West Java specialties, and granting them access to evaluate ecotourism activities. By demonstrating a willingness to receive feedback and suggestions, the management is fostering greater sustainability within the tourism industry.

The final phase emphasizes the importance of nurturing a solid rapport with tourists. To achieve this, Cipeujeuh Valley managers ought to present visitors with a diverse selection of enticing and exclusive tour packages. It is essential to supply pertinent information about Cipeujeuh Valley's tourism offerings via the website and social media outlets consistently to entice tourists. Moreover, captivating promotions can be disseminated through private messaging services to further stimulate interest.

Social media has a favorable impact on ecotourism as it effectively and efficiently promotes tourism products. Moreover, it functions as an online platform for tourists to share their experiences and obtain information about ecotourism [14].

3.4.5 Revenue Streams

The Cipeujeuh Valley, known for its numerous tourist attractions, has the potential to generate revenue from multiple sources. These include admission fees for entering the valley, individual tickets for specific attractions, the bundling of

Table 6. Cipeujeuh Valley's Channels

Channel type (Theory)		Channel type (Application)	
Owned	Directly	Web sales	Marketing via website and social media such as Instagram, Facebook, Twitter, etc
		Owned	Marketing with tourism map (print) attached to Clinic, Laundry, Catering and
Partner	Not directly	Partner	Islamic boarding school Community (Students, builder Teachers and All Ma'had Apparatus) MSMEs in the boarding school environment Alumni Association Student's parents Regional Government of Garut Regency Bureau or tourist tour service
		Tourists	Word of mouth

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attractions into comprehensive tour packages, rental services for camping equipment and other necessary items, and various other tourism-related services. Furthermore, the Economic Sector of Islamic Boarding School provides funding through loans. Moreover, the provision of insurance and certification services for various activities contributes to the revenue generated during tourist activities.

3.4.6 Key Resources

The presence of appealing natural resources, religious activities, infrastructure, and facilities that cater to the needs of tourists is crucial. Moreover, having human resources with a strong interest in tourism activities is a vital asset that can be continuously developed for the ecotourism of Cipeujeuh Valley. It is of paramount importance to maintain the preservation of natural resources for the purpose of achieving environmental sustainability by ensuring that all activities at tourist attractions are centered around environmental conservation and do not disrupt or harm the natural surroundings.

Local religious activities and wisdom are among the attractions that ought to be introduced to tourists to preserve the cultural and traditional heritage of the community. To this end, engaging tourists in activities, art performances, exploration, and direct interaction with knowledgeable individuals can be effective strategies. Additionally, the development and improvement of infrastructure and facilities can enhance the comfort and convenience of tourists during their visits to Cipeujeuh Valley.

Finally, it is essential for human resources involved in tourism activities to receive training and continuous support to maintain their enthusiasm and involvement in the sector. Establishing a competent workforce with expertise in managing tourism is crucial for ensuring the standardization of ecotourism services [15].

3.4.7 Key Activities

Cipeujeuh Valley is dedicated to developing nature tourism and tourism centered around the Islamic boarding community, which are both essential and require further growth. This approach represents a pioneering strategy to improve the appeal of Cipeujeuh Valley's tourism offerings. By offering more enticing attractions, visitors are likely to visit the area more frequently, ultimately leading to increased economic benefits for Cipeujeuh Valley. The primary activities available in Cipeujeuh Valley are summarized in Table 7.

The table outlined in Table 7 indicates that Cipeujeuh Valley's primary activities are education, sports, agrotourism, and camping. By incorporating environmental education into educational activities, young people's understanding of the environment can be enhanced, and environmental awareness can be fostered among a wide range of participants [16]. Moreover, transforming sports into a tourist attraction can have a positive impact on tourists by promoting physical and mental well-being through physical movement [17]. Developing agrotourism as a tourist attraction can provide multiple benefits, including stimulating scientific and educational activities, improving overall health and fitness, relieving stress and monotony, and offering access to organic food [18]. Camping, too, offers tourists a direct way to appreciate the beauty of nature and provides a recreational experience that immerses them in the thrill of outdoor living [19].

Table **7.** Cipeujeuh Valley's Key Activities

Key Activities	Area	Tourism Activities
Nature - Based Tourism	Cipeujeuh Valley Area and Rivers	Enjoy the view Agrotourism Camping Tree Guard The River Tube Gymnastics Cross the Bridge Jogging Archery Education planting Self-Service Culinary
Tourism Based on the Community of islamic boarding school	Darul Arqam Islamic Boarding School Area	Islamic History Tour West Java Art Show A sport show Visit to the creation of ecobrick, maggots, management trash

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Table 8. Cipeujeuh Valley's Key Resources

Cost Details	Key Resources		
	Infrastructure	**Facilities**	**Human Resources**
Fixed Cost	Construction of major infrastructure supporting tourism utilities, maintenance	Information center construction Plantation area development Setting campground area Procurement of plant seeds for agrotourism activities Promotional activities Other	Manager's Salary
Variable Cost	Overhead cost	Bridge repair Repair the sails Toilet repair Procurement of needs in tourist packages Promotional and gift programs	Training management for manager and the community of Islamic boarding schools

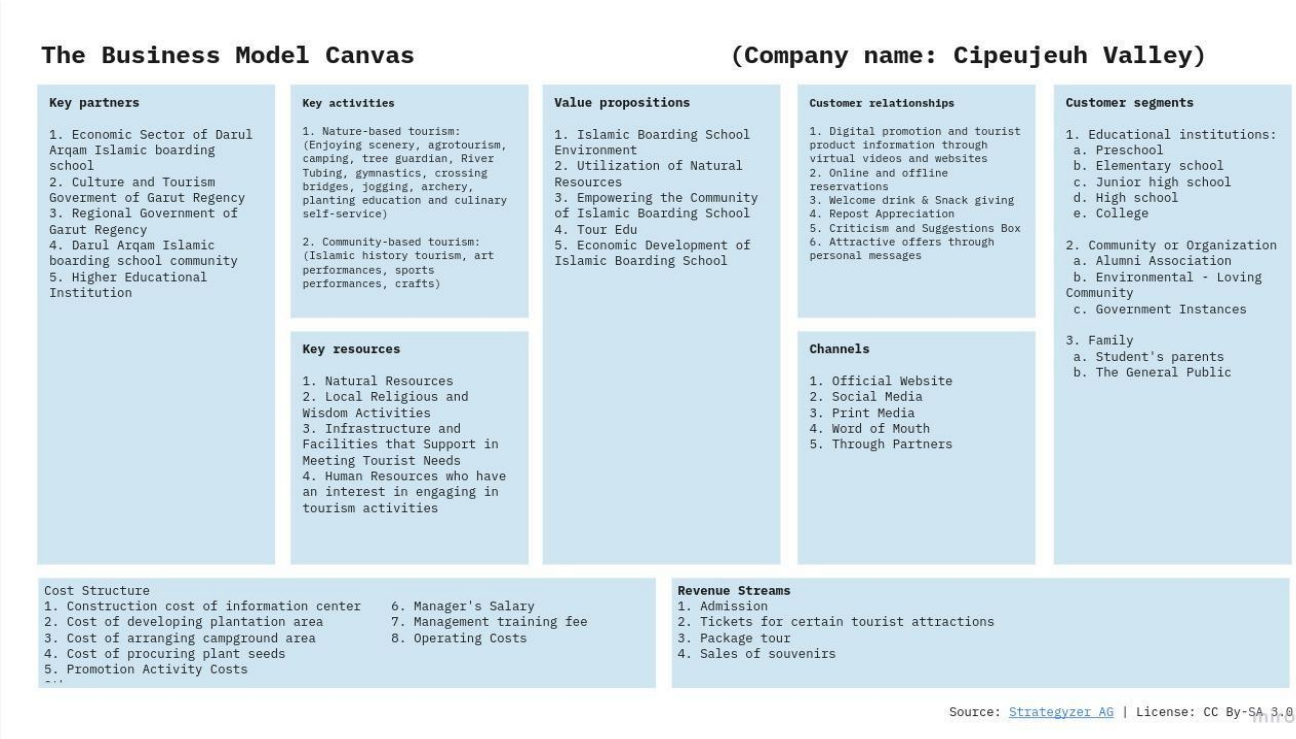

Figure **3**. Business Model Canvas

3.4.8 Key Partner

A key partner is a collaborative partner that plays a crucial role and possesses the potential to support each other in ongoing and future tourism endeavors. Cipeujeuh Valley, designated for development, requires the support and assistance of various stakeholders to progress in a positive direction. The Darul Arqam Islamic Boarding School, as a business sector, can assume the role of a manager responsible for the financial aspects of ecotourism development. Moreover, the Ministry of Culture and Tourism, operating under the Garut Regency Regional Government, can offer various forms of assistance and training throughout the development process. The Islamic Boarding School Community, as a party directly involved with the Cipeujeuh Valley, is also a crucial partner in the collaborative effort to manage and sustain the Cipeujeuh Valley area. Furthermore, higher education institutions are expected

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to consistently contribute to the discussion and consideration of development through their research endeavors. It is hoped that these entities will actively participate in the current and future tourism development activities within Cipeujeuh Valley.

3.4.9 Cost Structure

The expenses incurred in the development of Cipeujeuh Valley encompass fixed costs, which are recurring and variable costs and semi-variable costs, which are not routinely incurred but are only incurred under specific circumstances. The cost structure required for Cipeujeuh Valley is illustrated in Table 8.

Figure 3 displays the standard Business Model Canvas. To successfully integrate the nine key elements within the canvas, it is essential to initiate the optimization process for each element, whether they are absent or present and require improvement.

4. Conclusion

The calculation of the RZI value indicates that Cipeujeuh Valley's ecotourism falls within the intermediate range at 52.38%. Intermediate ecotourism typically involves small group visits using readily available transportation and taking advantage of the facilities and services provided by the tourism management. Four push factors for tourist visits are sports and adventure, recreation and relaxation, social status, and quality time. Nature and local community, also enjoying facilities serve as pull factors for tourists. Among the community, 61.3% have expressed their willingness to participate in the development of Cipeujeuh Valley's ecotourism. They are interested in activities such as maintaining and managing agrotourism gardens, acting as educational facilitators, guiding visitors during their tourism experiences, promoting Islamic history and the interpretation of the Quran and Hadith, as well as selling handicraft products. The business model for the development of Cipeujeuh Valley's ecotourism is depicted using the Business Model Canvas (BMC), targeting customer segments that include educational institutions, communities or organizations, and families. The value proposition revolves around the environment of the Islamic boarding school, sustainable utilization of natural resources, empowerment of the Islamic boarding school community, educational tourism, and economic development. Channels of promotion include official websites, social media, print media, word of mouth, and partnerships. Customer relationship consists of the acquisition phase, retain phase, and enhancement phase. Revenue streams are generated from admission tickets, attraction tickets, camping equipment rentals, and souvenir sales. Key resources include attractive natural resources, religious and local wisdom activities, infrastructure and facilities that cater to the needs of tourists, and a workforce with a keen interest in tourism activities. Key activities focus on nature-based and community-based tourism experiences. Key partners involve the Economic Sector of Darul Arqam Islamic Boarding School, the Garut Regency Local Government, the Darul Arqam Community, and Higher Education Institutions. The cost structure encompasses the construction of an information center, development of plantation areas, campground planning, plant seedling procurement, promotional activities, management salaries, training programs, and operational expenses.

The determination of the RZI value indicates that Cipeujeuh Valley's ecotourism falls within the intermediate range, at 52.38%. This type of ecotourism typically involves small group visits, utilizing readily available transportation, and making use of the facilities and services provided by the tourism management. The push factors for tourist visits are sports and adventure, recreation and relaxation, social status, and quality time. Nature and the local community, as well as the availability of facilities, serve as pull factors for tourists. Among the community, 61.3% have expressed their willingness to participate in the development of Cipeujeuh Valley's ecotourism. They are interested in activities such as maintaining and managing agrotourism gardens, acting as educational facilitators, guiding visitors during their tourism experiences, promoting Islamic history and the interpretation of the Quran and Hadith, as well as selling handicraft products. The business model for the development of Cipeujeuh Valley's ecotourism is depicted using the Business Model Canvas (BMC), targeting customer segments that include educational institutions, communities or organizations, and families. The value proposition revolves around the environment of the Islamic boarding school, sustainable utilization of natural resources, empowerment of the Islamic boarding school community, educational tourism, and economic development. Channels of promotion include official websites, social media, print media, word of mouth, and partnerships. Customer relationship management encompasses three key phases: acquisition, retain, and enhancement. Revenue is generated from various sources, such as admission and attraction tickets, camping equipment rentals, and souvenir sales. Key resources include attractive natural resources, local wisdom activities, infrastructure and facilities catering to tourists' needs, and a workforce with a keen interest in tourism activities. Key activities focus on nature-based and community-based tourism experiences. Key partners include the Economic Sector of Darul Arqam Islamic Boarding School, the Garut Regency Local Government, the Darul Arqam Community,

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Enhancing Lipid Extraction from *Chlorella vulgaris* Microalgae for Biodiesel Production: Application of Natural Deep Eutectic Solvent (NaDES) in Cell Disruption

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Abstract

Natural deep eutectic solvent (NaDES) pre-treatment offers a promising alternative strategy to enhance lipid extraction efficiency from microalgae by influencing the integrity of the cell wall, thereby improving solvent accessibility to the cytoplasm and facilitating the release of intracellular lipid content. In this study, *Chlorella vulgaris* biomass underwent pre-treatment with four different NaDES formulations, each based on choline chloride (ChCl) with varying molar ratios: ChCl : Glycerol (1:2), ChCl : Glycerol (1:1), ChCl : Oxalic Acid (1:2), dan ChCl : Oxalic Acid (1:1).We analyzed the impact of these NaDES pre-treatments on lipid yield and fatty acid profiles. The extracted lipids exhibited an acid value of 42.56 mg KOH/g fat and a free fatty acid content of 0.25%. Samples subjected to NaDES treatment showed significant increases in lipid extraction efficiency, with lipid yields ranging from 1.25 to 2.3 times higher than those of untreated biomass extracted using hexane ($p < 0.05$). The highest lipid yield was observed in samples treated with ChCl : Glycerol (1:1), achieving a total lipid yield of 19.44% (w/w), more than double that of the untreated biomass. Although minor variations in fatty acid profiles were noted due to the NaDES treatment, the dominant fatty acids in each variation remained palmitic acid (C16:0, 21-29.5%) and oleic acid (C18:1, 13-43.88%).

Keywords: *Chlorella vulgaris; Natural deep eutectic solvent; Pre-treatment; Cell disruption; Lipid extraction; Fatty acid profile*

1. Introduction

Energy is a pivotal necessity impacting various aspects of daily life, including fuel for machinery and transportation. Currently, 85% of global energy consumption relies on non-renewable resources, making the fuel industry vulnerable to energy crises [1]. Consequently, alternative energy from renewable resources is urgently needed. Biodiesel emerges as a promising renewable energy source, integrating a neutral carbon cycle and potentially addressing issues related to conventional diesel consumption.

Biodiesel can be produced using oils or lipids from organisms such as *Chlorella vulgaris*. Microalgae offer several advantages over conventional terrestrial biomass like corn, as they can be cultivated year-round, leading to higher biomass productivity, short growth cycles, high lipid yields, efficient CO_2 fixation, and minimal land usage without competing with food resources [2].

One major challenge in lipid extraction is the low yield due to the complex and rigid cell wall structure of microalgae, which hinders solvent interaction with intracellular lipids and necessitates large volumes of solvent [3]. Cell wall disruption is essential to maximize lipid yield. However, conventional disruption methods, such as chemical treatments with ionic liquids or physical methods like sonication, have drawbacks, including high energy consumption, high costs, complex processes, and environmental concerns [4, 5].

A promising alternative is the use of deep eutectic solvents (DES), environmentally friendly green solvents, as a pre-treatment to overcome the limitations of conventional cell disruption and solvent extraction methods. DES are simple to prepare, cost-effective, non-toxic, and biodegradable [6]. They are typically synthesized from organic salts like choline chloride or choline acetate and hydrogen bond donors such as carboxylic acids, amides, or amines to form eutectic mixtures with lower melting points than their individual components [7]. Natural deep eutectic solvents (NaDES) are a subset of

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DES synthesized from compounds found in plant primary metabolites, such as organic acids, sugars, and choline derivatives [8].

NaDES can influence the hydrogen bonds within the microalgae cell wall, thereby affecting its integrity and permeability [3]. Factors influencing the efficiency of NaDES in extraction or cell disruption include the ratio of organic salt to hydrogen bond donor, the microalgae strain, and the amount of deionized water added for aqueous DES. This study aims to analyze the impact of NaDES application on the microalgae *Chlorella vulgaris* to achieve efficient lipid production with high yields for biodiesel precursors.

2. Methodology

2.1. Microalgae culture

The microalgae culture used in this research was *Chlorella vulgaris*, obtained from The Laboratory of Indonesian Culture Collection (InaCC) in Cibinong. The cultivation medium was commercial NPK fertilizer (Nutricomp-D), which contains 22% total nitrogen (N), 17% phosphorus pentoxide (P_2O_5), 15% potassium oxide (K_2O), and 12.5% magnesium oxide (MgO). Other chemicals for NaDES synthesis, such as choline chloride (ChCl), glycerol, and oxalic acid, were sourced from Merck and HiMedia.

2.2 Synthesis of Natural Deep Eutectic Solvents (NaDES)

NaDES were synthesized using choline chloride (ChCl) as the hydrogen bond acceptor (HBA) and glycerol or oxalic acid as the hydrogen bond donors (HBD). The molar ratios of the mixtures were varied as 1:1 and 1:2 (HBA:HBD) for each NaDES variation. ChCl, glycerol, and oxalic acid were weighed according to the respective molar ratios and added to an Erlenmeyer flask. The mixtures were stirred at 80°C using a magnetic stirrer until a colorless solution was obtained (after 1-2 hours). The NaDES solutions were then stored in an oven at 50°C for 24 hours to prevent water absorption and subsequently kept in tightly closed falcon tubes in a desiccator before use. Before use in the extraction process, NaDES (ChCl-G and ChCl-OA) were mixed with 40% (v/v) deionized water to decrease viscosity and enhance mass transfer.

2.3 Biomass Pre-treatment with NaDES

Dried *Chlorella vulgaris* biomass was pre-treated with NaDES molar variations: ChCl-G (1:1), ChCl-G (1:2), ChCl-OA (1:1), and ChCl-OA (1:2). The dried microalgae were mixed with NaDES at a solvent-to-biomass ratio of 5 mL/g. Pre-treatment was conducted at room temperature (25-27°C) for 24 hours with constant stirring using a magnetic stirrer. Post-treatment, NaDES and biomass were separated via centrifugation at 6,000 rpm for 20 minutes. The supernatant containing some lipids mixed with NaDES was stored in a desiccator before GC-MS analysis. The pre-treated biomass was washed with deionized water and centrifuged four times at 6,000 rpm for 10 minutes each. The biomass pellet was dried in an oven at 60°C for 24 hours, then ground into a powder using a mortar and pestle for further analysis.

2.4 Lipid Extraction using Bligh-Dyer and Hexane Methods

Lipid extraction from dried microalgae biomass was performed using the Bligh & Dyer method (1959) as a control to compare traditional solvent extraction (chloroform-methanol) with the NaDES pre-treatment followed by hexane extraction [9]. This control method helps evaluate the effectiveness of NaDES in improving lipid yield relative to a widely used conventional extraction technique. Dried biomass (1 gram) was mixed with 60 mL of chloroform and methanol (1:2 v/v) in a Soxhlet system for 5 hours at 70°C. Following this, 1 mL of chloroform and 1 mL of water were added to the solution and vortexed for 1 minute. The mixture was centrifuged at 5,000 rpm for 8 minutes, separating the organic phase containing lipids and chloroform. Dried pre-treated microalgae with NaDES were also extracted using the same method but with hexane as the solvent. NaDES pre-treated microalgae were extracted using hexane, chosen for its relevance to industrial lipid extraction. The Bligh-Dyer method (chloroform-methanol) was used as a control to compare traditional and industrially relevant solvents. Both methods provide comparable fatty acid profiles for GC-MS analysis, validating the comparison. The mixture of microalgae lipid and solvent was evaporated on a water bath at 60°C for approximately 2 hours to obtain the lipid content. Lipid yield was determined using the following equation:

$$Y_{lipid} = \frac{W_{lipid}}{W_{dry\ biomass}} \text{ x } 100\% \qquad (1)$$

in which Y_{lipid} is lipid yield (% w/w), W_{lipid} is extracted lipid weight (gr), and W_{dry} is the weight of dried biomass (gr).

2.5 Determination of Acid Value and Free Fatty Acid Content

The acid value and free fatty acid (FFA) content were determined using the titration alkalimetry method by a nationally accredited reference laboratory. Lipid samples (0.3 g) were weighed into a 250 mL Erlenmeyer flask and diluted in ethanol solvent, with 3 drops of phenolphthalein (PP) indicator added. The prepared sample was titrated against 0.02 N KOH solution until a pink color persisted for 30 seconds. The acid value was calculated using the following equation:

$$AV = \frac{V_{titration}\ x\ Mr_{KOH}\ x\ N_{KOH}}{W_{sample}} \qquad (2)$$

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In which AV is acid value, $V_{titration}$ is titration volume (ml), Mr_{KOH} is the molecular weight of KOH which is 56,11 gr/mol, NKOH is KOH concentration which is 0,02 N, and W_{sample} is lipid weight (gr).

Free fatty acid (FFA) content was considered as palmitic acid. Lipid samples (0.5 g) were weighed and diluted with ethanol in a 250 mL Erlenmeyer flask, with 3 drops of PP indicator added. The prepared sample was titrated against 0.02 N NaOH solution until a pink color persisted for 30 seconds. The FFA content was calculated using the following equation:

$$FFA = \frac{V_{titrasi}\ x\ Mr_{PA}\ x\ N_{NaOH}}{W_{sampel}\ x\ 1000}\ x\ 100\% \quad (3)$$

In which FFA is free fatty acid content (%), $V_{titrasi}$ is titration volume (mL), Mr_{PA} is palmitic acid molecular weight which is 256 gr/mol, NNaOH is NaOH concentration which is 0,05 N, dan W_{sample} is lipid weight (gr).

2.6 Fatty Acid Components Analysis

The fatty acid profile of *Chlorella vulgaris* lipid was analyzed using gas chromatography-mass spectrometry (GC-MS) with a flame ionization detector (FID) by a nationally accredited reference laboratory. The lipid extract used for GC-MS analysis was obtained from the pooled extract of both the colloidal phase and the biomass extract. Lipid samples were filtered using a 0.22 µm syringe filter. The stationary phase was cyanopropyl on a DB FastFame capillary column. The injector temperature was set at 300°C, and the detector temperature was 250°C. Argon gas was used as the mobile phase with a flow rate of 1 mL/min. The oven column's initial temperature was 190°C for 5 minutes, followed by a heating rate of 10°C/min until 250°C. The temperature was maintained at 250°C for 5 minutes, then increased to 280°C at a heating rate of 10°C/min, held for 30 minutes, and finally cooled to room temperature.

2.7 Statistical Analysis

Experiments were conducted in duplicate, and mean values were reported. Data processing was performed using Microsoft Excel (Office Enterprise 2019) with single-factor analysis of variance (ANOVA) to determine statistical significance.

2.8 Manuscript Preparation

In preparing this manuscript, the authors utilized several AI-based tools to enhance the quality and clarity of the writing. ChatGPT4.0 was employed to improve language clarity and ensure concise, precise communication of scientific concepts. For efficient and accurate reference management, Zotero was used to organize and cite all sources. Grammarly assisted in refining the English language and ensuring adherence to proper grammar and style, which is crucial for clear communication with an international audience. Additionally, QuillBot was applied for paraphrasing certain sections, helping to maintain originality while preserving the intended meaning of rephrased content.

3. Results and Discussion

3.1. Chlorella vulgaris *Microalgae Growth Curve and Biomass Productivity Analysis*

The growth curve of *Chlorella vulgaris* biomass is presented in Figure 1, showing multiple growth phases: lag, log (exponential), stationary, and death phases. The lag phase occurred after biomass inoculation until the third day of cultivation, during which microalgae acclimatized to new conditions such as medium pH, temperature, and light [10].

A slight decrease in biomass was observed on the first day after inoculation, with cell density dropping by 1% compared to the immediate post-inoculation value. Generally, biomass decline for *Chlorella vulgaris* during the lag phase ranges from 3.81% to 4.5% [10]. In this study, the cell decline was lower than literature values, possibly due to the use of

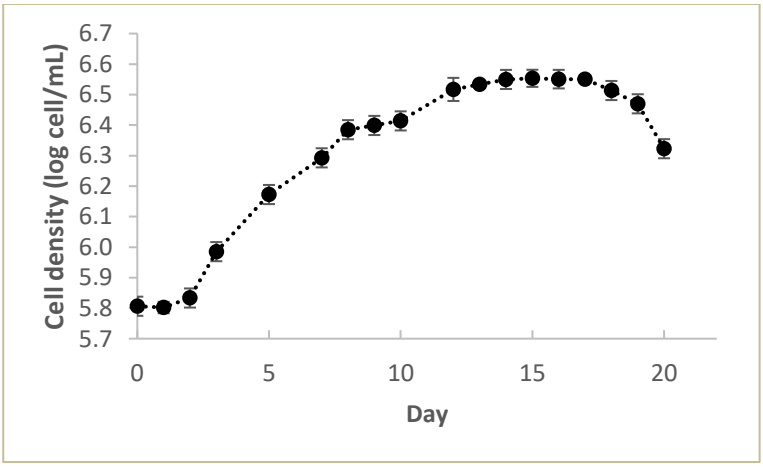

Figure 1. *Chlorella vulgaris* biomass growth curve

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similar medium and cultivation conditions, facilitating faster acclimatization.

The log phase, characterized by active cell division and biomass increment, spanned from day 3 to day 13. The specific growth rate was determined based on biomass at the beginning and end of the log phase. The stationary phase, where growth and death rates equalize, occurred from day 13 to day 17, with maximum biomass concentration of 0.057 g/L and cell density of 3.54 x 10^6 cells/mL reached on day 14. The death phase from day 18 to day 20 was marked by a drastic decline in cell density due to reduced nutrient availability, dissolved oxygen, and potential contamination [11].

The specific growth rate (μ) of *Chlorella vulgaris* in this study was 0.127 day^{-1}, with a doubling time of 5.433 days. Literature reports a specific growth rate of 0.344 day^{-1} after 7 days of cultivation with a maximum cell density of 1.88 x 10^7 cells/mL using pure CO_2 aeration [12]. Variations in growth rates are attributed to differences in microalgae strain, medium-induced stress, and cultivation conditions.

Pure CO_2 aeration, for instance, increases CO_2 fixation rates, thereby enhancing metabolic reactions and doubling the growth rate compared to regular aeration (0.04% CO_2) [13]. This study's doubling time of 5.433 days for *Chlorella vulgaris* with regular aeration contrasts with the 2.015 days reported for pure CO_2 aeration [12].

Biomass productivity in this study was 0.004 $g \cdot L^{-1} \cdot day^{-1}$, lower than the general biomass productivity range of 0.025 - 0.040 $g \cdot L^{-1} \cdot day^{-1}$ reported in other studies [14, 15]. Differences in cultivation treatments, such as medium composition, contribute to this variation. In literature, media like F2 and Nutriverde with higher nitrogen concentrations (9.29% and 5.571%, respectively) were used, while the NPK Nutricomp-D commercial fertilizer used in this study contained only 2.2% nitrogen. Thus, lower nitrogen availability in this study led to lower maximum biomass and productivity.

3.2. Lipid Character Analysis of Chlorella vulgaris Based on Acid Value and Free Fatty Acid Content

Analyzing the lipid characteristics of *Chlorella vulgaris* is crucial for determining its suitability for biodiesel production. This study evaluated lipid character based on acid value and free fatty acid (FFA) content. The acid value indicates the amount of potassium hydroxide (KOH) required to neutralize free fatty acids in the lipid. The acid value in this study was 42.56 mg KOH/g lipid.

Animal fats and vegetable oils used as biodiesel precursors typically have acid values between 50 - 200 mg KOH/g [16, 17, 18]. *Chlorella vulgaris* lipids generally have acid values ranging from 25 - 130 mg KOH/g [17, 18], aligning with the values observed in this study. However, the acid value remains high compared to biodiesel standards, indicating potential corrosiveness towards materials like aluminum, steel, and nickel [18]. High acid values can also lead to saponification during transesterification, reducing biodiesel yield and complicating product separation [18].

International standards such as ASTM D6751 and EN14214 require acid values below 0.5 - 0.8 mg KOH/g for biodiesel. Post-transesterification, biodiesel typically exhibits lower acid values, such as 0.6 mg KOH/g, indicating the potential for reduced acid values following transesterification and purification processes [19].

FFA content, indicative of the lipid's energy reserve in microalgae cells and formed through triglyceride hydrolysis, influences the lipid's oxidative stability and potential for biodiesel conversion. High FFA content suggests susceptibility to oxidation and potential clogging of machinery fuel filters, especially at lower temperatures [20]. Biodiesel production generally requires lipids with FFA content below 0.5% (w/w) [21].

In this study, the FFA content of *Chlorella vulgaris* lipid was 0.25% (w/w), indicating compatibility with biodiesel production conditions. The cultivation treatment used in this study resulted in lower FFA content compared to other studies, which reported FFA contents ranging from 0.5 - 6.9% (w/w) [21, 22, 23]. The lower FFA content observed may be attributed to the use of NPK Nutricomp-D, a minimal medium with reduced nitrogen concentration (0.158 g/L) compared to Bold Basal Medium (BBM) used in other studies (0.25 g/L). This reduced nitrogen concentration likely shifted the lipid composition towards higher triglyceride content and lower FFA levels, which is more suitable for biodiesel production [24].

3.3. Analysis of NaDES Application Influence on Microalgae Chlorella vulgaris *Lipid Yield*

The efficiency of lipid extraction from microalgae can be significantly affected by the solvent and pre-treatment methods used. In this study, the application of natural deep eutectic solvent (NaDES) as a pre-treatment method showed a positive impact on lipid yield from *Chlorella vulgaris*. Multiple variations of NaDES, consisting of choline chloride (ChCl) combined with glycerol (ChCl-G) or oxalic acid (ChCl-OA), and varying molar ratios, were tested.

The total lipid yield included lipids from both the colloid phase and the dry biomass post-NaDES pre-treatment. When the biomass was agitated in NaDES solution for 24 hours, lipids were released from the microalgae cells into the NaDES phase due to the cell disruption process. Additionally, since NaDES also functions as a liquid solvent, it facilitated lipid extraction during the disruption process. The lipid content extracted during the disruption was dispersed and mixed with the NaDES, forming the colloid phase.

Subsequent drying and extraction of the biomass post-pre-treatment using hexane yielded further lipids, contributing to the total lipid yield. Figure 2 illustrates the total lipid yields for each NaDES variation.

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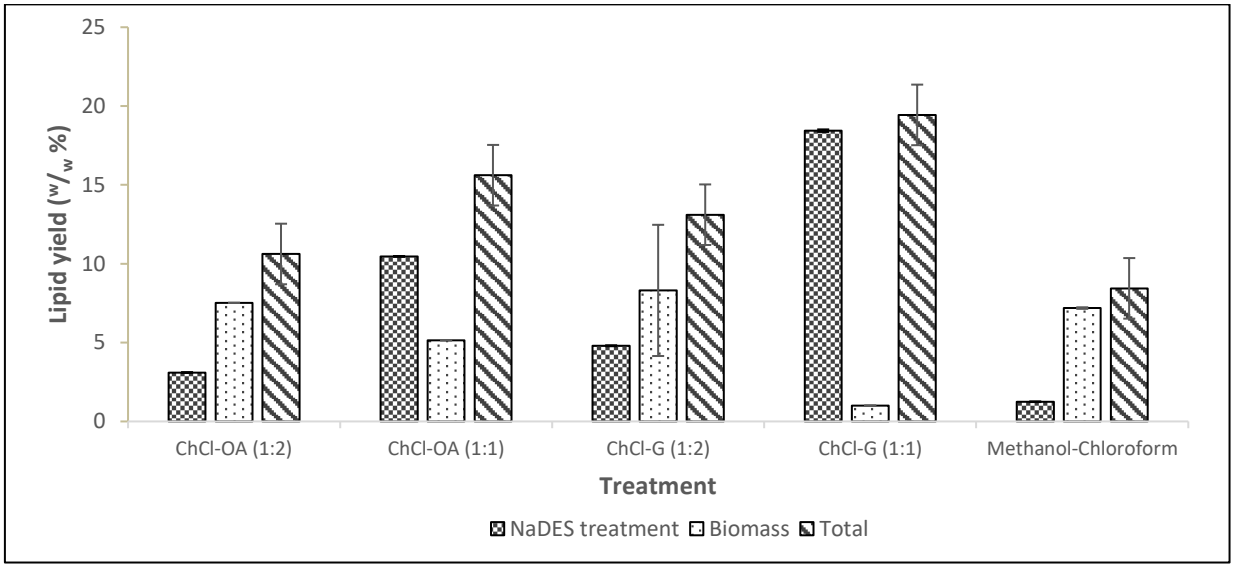

Figure 2. Lipid yield from the colloidal phase of NADES pre-treatment and dried biomass

The application of NaDES as a pre-treatment method significantly improved total lipid yields compared to untreated biomass, which had a total lipid yield of 8.44% (w/w). The highest total lipid yield, achieved using the NaDES mixture of ChCl and glycerol with a 1:1 molar ratio, reached 19.44% (w/w), more than doubling the yield from untreated biomass. In contrast, the lowest lipid yield was observed with the NaDES mixture of ChCl and glycerol at a 1:2 molar ratio. Overall, every NaDES and molar ratio variation experienced an increase in lipid yield compared to the untreated sample, demonstrating the effectiveness of NaDES pre-treatment in enhancing lipid extraction efficiency.

Literature indicates that NaDES pre-treatment with ChCl-OA (1:2), as well as urea and acetamide (1:2), could increase lipid yields by up to 80.90% and 75.25%, respectively, compared to untreated biomass [3]. The statistically significant p-values ($p < 0.05$) for all NaDES and molar ratio variations support the hypothesis that NaDES application can enhance lipid yield and extraction efficiency in microalgae *Chlorella vulgaris*.

NaDES solution is synthesized by combining the hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD). This reaction not only forms hydrogen bonds within the NaDES but also allows these components to interact with the microalgae cell wall, facilitating hydrogen bonding there as well [25]. This interaction enhances the rigidity and integrity of the cell wall, thus improving the release of intracellular lipids due to increased cell wall permeability—a result of the energy reduction in hydrogen bond interactions with macromolecules and hemicellulose [3, 7]. Consequently, NaDES application significantly boosts the efficiency of the lipid extraction process by improving solvent access to intracellular lipids [26].

The hydroxyl and carboxyl groups in NaDES form bonds with similar groups in the cell wall, impacting the hydrogen bonds between cellulose and hemicellulose that typically confer rigidity to microalgae cells [25]. Continuous stirring for 24 hours during the treatment also assists in the cell disruption process, enhancing the solvent's access to the cell wall and facilitating interaction with intracellular lipids.

According to this study, NaDES formulations comprising choline chloride (ChCl) and glycerol yielded higher total lipid outputs compared to those containing ChCl and oxalic acid. This variation in lipid yield may be attributed to differences in solvent viscosity. Generally, NaDES is characterized by its high viscosity, particularly evident in mixtures of ChCl and oxalic acid, which are composed of two solid compounds. The viscosity of the NaDES ChCl-OA (1:2) is 212.9 mm²/s, which is significantly higher compared to NaDES ChCl-G (1:1) at 52.3 mm²/s [27]. The elevated viscosity in the ChCl-OA mixture could restrict the solubility of microalgae during the extraction process, making it more challenging for the solvent to interact with the microalgae cell surface due to increased surface tension [28].

Comparative analysis of NaDES formulations shows that those based on ChCl and glycerol yield higher total lipid outputs compared to those with ChCl and oxalic acid, likely due to differences in solvent viscosity. The addition of 40% deionized water was employed to lower the viscosity of NADES, as high viscosity can hinder mass transfer during extraction. By reducing the viscosity, the NADES mixture became less resistant to flow, which allowed for improved solvent penetration into the microalgae cell wall. This enhanced solvent access to intracellular lipids, thereby increasing the overall extraction efficiency. However, care was taken to ensure that the addition of water did not dilute the NADES excessively, as this could disrupt the eutectic structure and reduce its efficacy as a cell wall disruptor. Adjusting the NaDES viscosity by adding deionized water reduces this effect, although excessive water can disrupt

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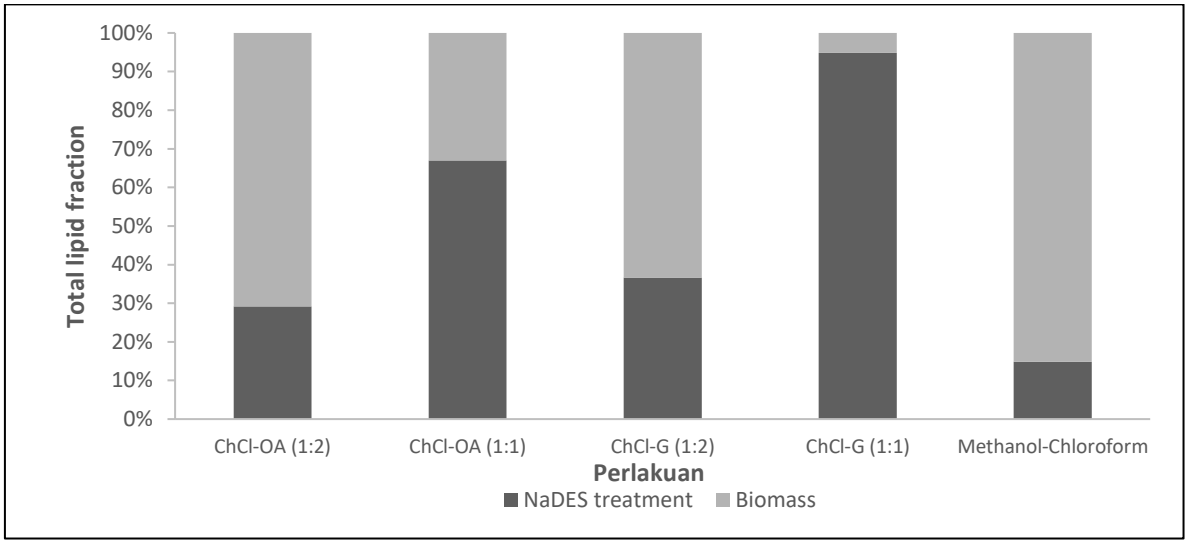

Figure 3. Distribution of lipid fraction from colloid phase and dried biomass phase of *Chlorella vulgaris*

the hydrogen bonding in NaDES, potentially degrading its effectiveness [28].

The molar ratio of HBA to HBD significantly influences the lipid yield, with a 1:1 ratio proving most efficient. This efficiency is reflected in the lipid yields from both the colloid and dried biomass phases. Notably, the highest lipid yield from the colloid phase was observed with NaDES ChCl-G (1:1), indicating superior permeability enhancement of the microalgae cell wall [27]. In contrast, the lipid yield from dried biomass after NaDES treatment shows variability, suggesting that further extraction from the biomass is necessary even after initial NaDES treatment.

The lipid yields from the colloid phase and dried biomass exhibit distinct variations across different NaDES treatments. The highest lipid yield from the colloid phase was observed in the NaDES formulation of ChCl-G (1:1), achieving 18.44% (w/w). This high yield underscores the effectiveness of ChCl-G in enhancing the permeability of microalgae cell walls, thereby facilitating the release of lipid content into the solvent during treatment. In contrast, the lowest lipid yield in the colloid phase, at only 1.25% (w/w), was found in the untreated samples.

The lipid yield from dried biomass after pre-treatment also concur some differences for each variation. The highest lipid yield is found in ChCl-G (1:2), which is 8,31% (w/w). Meanwhile, the lowest lipid yield is found on ChCl-G (1:1), which is 1% (w/w). The lipid yield from biomass shows that after NaDES pre-treatment, that the dried biomass potentially still has lipid content even though the cell wall has experienced lysis. Therefore, further biomass extraction after NaDES pre-treatment is still needed.

There are observable trends in the distribution of lipid yields between the colloid phase and dried biomass. Samples treated with NaDES generally exhibit higher lipid yields from the colloid phase, as demonstrated in Figure 3. This trend suggests that during NaDES application, intracellular lipids are effectively mobilized from the cell wall matrix into the solvent under continuous mixing conditions. Although methanol-chloroform was used as a control without NADES pre-treatment, a small colloidal phase was observed. This is likely due to the presence of some emulsified lipids in the solvent, which formed a colloidal suspension during the extraction process. The phenomenon has been noted in other studies where organic solvent extraction can result in the formation of colloidal phases even without prior cell disruption. The methanol-chloroform extraction method yielded the lowest lipid recovery, likely due to its inability to disrupt the rigid cell wall of *Chlorella vulgaris* as effectively as the NADES pre-treatment. While methanol-chloroform is a standard solvent for lipid extraction, it does not promote cell wall permeability, resulting in lower lipid release compared to the enhanced extraction achieved with NADES, which facilitates cell disruption

Moreover, it is evident that when a majority of lipids are released during NaDES pre-treatment, the residual lipid content in the dried biomass is reduced. This observation is supported by a strong negative correlation coefficient of -0.90 between lipid yields from the biomass and those from the colloid phase [3, 27]. This correlation indicates that higher lipid yields in the colloid phase correspond to lower yields in the dried biomass. This relationship is also noted in other studies [3], which report that the decline in lipid content from dried pre-treated biomass is attributable to the migration of lipids from the cell membrane into the NaDES aqueous phase.

3.4. NaDES Application Effect Towards Fatty Acid Profile of Microalgae Chlorella vulgaris *Biomass*

The quality of biodiesel derived from the transesterification of microalgae lipids is largely influenced by the characteristics of the triglycerides, which depend on the distribution and composition of the fatty acids in the lipid. The transesterification reaction, which converts lipids into fatty

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Table 1. Fatty acid components of lipid from colloid phase and dried biomass

Fatty Acid	Fatty Acid Components (% w/w)					
	ChCl-OA (1:2)	ChCl-OA (1:1)	ChCl-G (1:2)	ChCl-G (1:1)	*Untreated*	Biomass ChCl-G (1:2)
C12:0	-	0,69 ± 0,005	-	0,77 ± 0,014	-	-
C14:0	0,07 ± 0,00	0,38 ± 0,005	-	0,53 ± 0,013	-	-
C16:0	0,92 ± 0,022	3,18 ± 0,055	1,05 ± 0,00	4,35 ± 0,061	0,286 ± 0,004	2,42 ± 0,030
C16:1	-	-	-	-	-	1,02 ± 0,013
C18:0	0,27 ± 0,005	0,74 ± 0,013	0,35 ± 0,008	1,13 ± 0,016	0,17 ± 0,001	0,20 ± 0,005
C18:1	1,29 ± 0,028	4,38 ± 0,078	2,11 ± 0,049	7,79 ± 0,144	0,49 ± 0,012	1,49 ± 0,035
C18:2	0,45 ± 0,011	1,11± 0,019	1,10 ± 0,023	2,20 ± 0,044	0,15 ± 0,00	2,86 ± 0,055
C18:3	-	-	0,19 ± 0,004	1,64 ± 0,028	0,16 ± 0,002	2,51 ± 0,042
C20:2	-	-	-	-	-	0,75 ± 0,016
MUFA	1,29 ± 0,028	4,38 ± 0,078	2,11 ± 0,049	7,79 ± 0,1442	0,49 ± 0,012	2,51 ± 0,0221
PUFA	0,60 ± 0,014	1,11 ± 0,019	1,30 ± 0,027	3,84 ± 0,028	0,31 ± 0,002	6,15 ± 0,0419
TOTAL	3,15 ± 0,07	10,47 ± 0,18	4,80 ± 0,090	18,40 ± 0,320	1,25 ± 0,020	11,25 ± 0,17

acid methyl esters (FAME) or biodiesel, does not alter the fatty acid composition of the lipid precursors. Consequently, the fatty acid components play a critical role in determining the biodiesel's quality, affecting properties such as cetane number and cold flow [29].

Fatty acid composition is analyzed using gas chromatography-mass spectrometry (GC-MS), with results for each variation displayed in Table 1. The fatty acid content, expressed as a percentage of the dry biomass weight, is sampled from the colloid phase after NaDES pre-treatment. Additionally, pre-treated dried biomass samples, particularly for the ChCl-G (1:2) variation, are analyzed for fatty acid content.

Detected fatty acid types range from C12 to C20. Predominantly, the fatty acid composition is dominated by C16 (palmitic acid) and C18 (stearic, oleic, linoleic, and linolenic acids). This dominance is consistent with other studies, which frequently report high concentrations of C16 and C18 fatty acids in *Chlorella vulgaris* [3]. The high content of these fatty acids is attributed to their solubility in the solvents used, which facilitates greater extraction efficiency compared to other fatty acids.

Table 2 illustrates the fraction distribution for each fatty acid relative to the total lipid content, providing a comparison with literature references for untreated biomass. This data reveals that C16 and C18 fatty acids contribute to 80-90% of the total fatty acid content, significantly influencing the biodiesel's final characteristics and quality. The highest fraction distribution of C16 is observed in the colloid phase of the NaDES ChCl-OA (1:1) variation, at 30.35%, while the highest distribution of C18 is found in the colloid phase of ChCl-G (1:2), at 74.13%.

The prominent presence of C18 and C16 fatty acids is advantageous as they enhance fuel quality and ignition properties. Additionally, a high concentration of C18 fatty acids contributes to greater oxidative stability during storage. Saturated fatty acids, such as palmitic and stearic acid, are associated with higher cetane numbers, suggesting that the NaDES ChCl-OA (1:1) colloid phase sample exhibits significant potential as a biodiesel precursor, meeting the EN14214 standard's minimum cetane number requirement of 51. In contrast, untreated biomass samples contain longer fatty acid chains like C22:6 and C20:5, which are absent in the studied samples and are known to adversely affect biodiesel quality by lowering the cetane number and promoting fuel oxidation [30].

Figure 4 provides a comparison of the fraction distribution of polyunsaturated fatty acids (PUFA), monounsaturated fatty acids (MUFA), and saturated fatty acids (SFA) for each variation. The ChCl-G (1:2) biomass sample exhibits a higher PUFA distribution, leading to a lower cetane number and higher iodine number, indicating potential issues with smoking upon ignition and reduced oxidation stability. Conversely, the ChCl-OA (1:1) colloid phase sample, with a lower PUFA distribution, is likely to exhibit higher oxidation stability and a lower iodine number, aligning more closely with international biodiesel standards, which limit C18:3 distribution to a maximum of 12% to avoid undesirable properties in biodiesel precursors.

The lipid composition rich in long-chain saturated fatty acids typically leads to a lower cold filter plugging point, causing precipitation and potential filter clogging. However, this study did not detect long saturated fatty acid chains. It is observed that the colloid phase samples ChCl-G (1:2) and ChCl-OA (1:1) have a higher distribution of MUFA, particularly C18:1 (oleic acid), suggesting that these lipids

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Table 2. Fraction distribution for each fatty acid contents

Fatty Acid	Fraction Distribution for Each Fatty Acids (%)						
	ChCl-OA (1:2)	ChCl-OA (1:1)	ChCl-G (1:2)	ChCl-G (1:1)	Untreated	Biomass ChCl-G (1:2)	Biomass untreated [35, 36]
C4:0	-	-	-	-	-	-	0,19 %
C6:0	-	-	-	-	-	-	0,14%
C8:0	-	-	-	-	-	-	0,13%
C10:0	-	-	-	-	-	-	0,19%
C12:0	-	6,58%	-	4,18%	-	-	
C14:0	2,21%	3,61%	-	2,85%	-	-	1,3%
C15:0	-	-	-	-	-	-	0,19%
C16:0	29,19%	30,35%	21,84%	23,62%	22,88%	21%	29,52%
C16:1	-	-	-	-	-	9%	1,88%
C17:0	-	-	-	-	-	-	0,24%
C17:1	-	-	-	-	-	-	6,00%
C18:0	8,53%	7,04%	7,24%	6,12%	13,38%	2%	2,74%
C18:1	40,90%	41,80%	43,88%	42,35%	39,22%	13%	17,01%
C18:2	14,41%	10,63%	23,01%	11,95%	11,83%	25%	27,74%
C18:3	-	-	4,03%	8,92%	12,69%	22%	11,5%
C20:2	-	-	-	-	-	7%	-
C20:4	5%	-	-	-	-	-	-
C20:5	-	-	-	-	-	-	0,13%
C22:6	-	-	-	-	-	-	0,13%
SFA	39,93%	47,57%	29,03%	36,98%	36,26%	23%	64,39
UFA	60,07%	52,43%	70,92%	63,22%	63,74%	77%	35,61

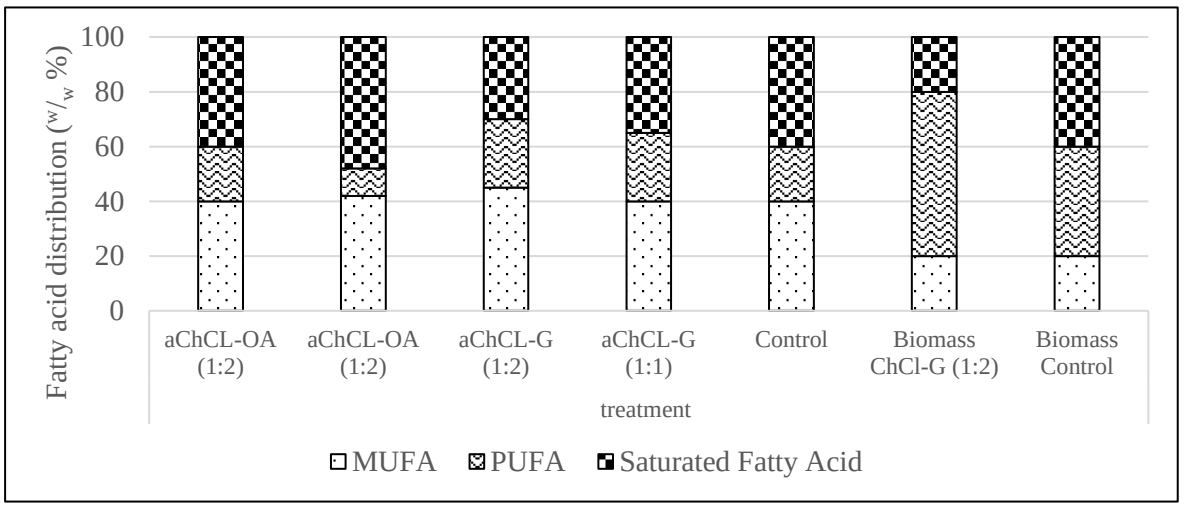

Figure 4. Omega fatty acid content in lipid of *Chlorella vulgaris*

may possess favorable general biodiesel characteristics and are likely to meet global biodiesel standards. Therefore, NaDES pre-treatment could potentially aid in ensuring compliance with standard biodiesel properties, enhancing the viability of microalgae-derived biodiesel.

According to the GC-MS results, the extracted lipid from *Chlorella vulgaris* includes significant quantities of omega fatty acids, as detailed in Figure 5. The omega fatty acid content within these lipids shows promising potential for use as pharmaceutical feedstocks, such as health supplements. This is particularly relevant for applications where the fatty acid characteristics are less suitable for biodiesel production. The omega fatty acids present, specifically omega-3, omega-6, and omega-9, are essential fatty acids that cannot be synthesized by the human body and are predominantly derived from polyunsaturated fatty acids (PUFAs), making them highly valuable for supplement production in the pharmaceutical industry [31].

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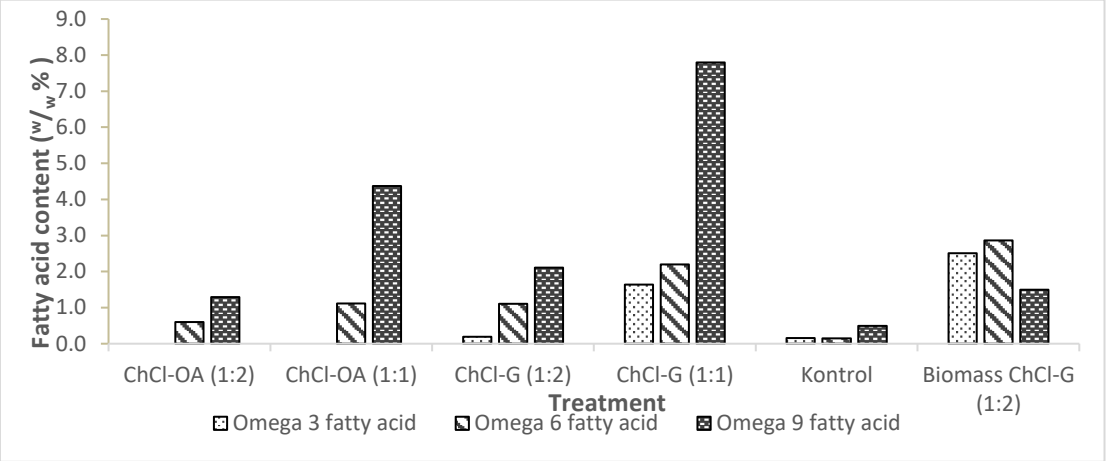

Figure 5. Omega fatty acid content in lipid of *Chlorella vulgaris*

In particular, the lipid samples from the dried biomass variation ChCl-G (1:2) and the colloid phase ChCl-G (1:1) exhibit the highest contents of omega fatty acids. The omega-6 content in these variations is measured at 2.86% and 2.199% respectively, while omega-3 content is found to be 2.51% and 1.6416% respectively. These concentrations indicate a robust potential for these specific lipid extracts to be developed into nutritional supplements, providing an alternative application for microalgae lipids that extends beyond energy production into health and wellness sectors.

The colloid phase sample of ChCl-OA (1:2) exhibits a lower overall omega fatty acid content but notably includes C20:4 omega-6, a long-chain fatty acid. Long-chain fatty acids, while beneficial in various applications, are not ideal as biodiesel precursors due to their impact on fuel properties such as viscosity and cold flow behavior. Consequently, this specific lipid composition makes the ChCl-OA (1:2) sample more suitable for pharmaceutical applications, particularly in the production of omega supplements, where long-chain omega-6 fatty acids are highly valued for their health benefits.

Conversely, the colloid phase sample of ChCl-G (1:1) contains higher contents of omega-3 and omega-6 fatty acids, which, combined with a higher monounsaturated fatty acid (MUFA) and omega-9 content, makes it better suited as a biodiesel precursor. The presence of MUFAs and omega-9 fatty acids enhances the lipid profile for biodiesel production by improving the fuel's oxidative stability and lowering its cloud point, thus meeting more of the biodiesel industry standards.

4. Conclusion

This study demonstrates that pre-treatment with natural deep eutectic solvents (NaDES) can significantly enhance the efficiency of lipid extraction from *Chlorella vulgaris*. The application of NaDES disrupts the cell wall, facilitating easier access for solvents to interact with intracellular lipids. As a result, the total lipid yield from the biomass treated with NaDES varies depending on the specific NaDES formulation and molar ratio used. The highest total lipid yield, amounting to 19.44% (w/w), was observed with the NaDES variation of choline chloride (ChCl) and glycerol at a 1:1 molar ratio. Gas chromatography-mass spectrometry (GC-MS) analysis further reveals variations in the fatty acid profiles of the extracted lipids, with a predominance of C16 (palmitic acid) and C18 (stearic and oleic acids) fatty acid chains. These findings underscore the potential of NaDES pre-treatment not only to improve lipid yield but also to influence the fatty acid composition, which is crucial for subsequent applications such as biodiesel production.

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Biomonitoring of Air Quality Using Lichen as Bioindicator in The Greater Bandung Area, West Java

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Abstract

Air quality assessment in an area can be determined by conducting biomonitoring using bioindicator organisms, including lichen. Lichen is a symbiotic organism that passively absorbs nutrients and chemical compounds from the air. This research was conducted in the Greater Bandung area, including Bandung City and West Bandung Regency, to observe the abundance of lichen species so that the purity of the atmosphere could be evaluated at each research location and determine the effect of environmental variables on lichen abundance.The survey area was divided into 40 units spread over two locations in Bandung, namely Djuanda street (JD), Kebon Kawung street (KK), and two locations in West Bandung Regency, namely Padalarang street (PD) and Curug Cimahi (CC). CC locations with low levels of pollution were used as comparison areas. Lichen samples from observation locations were identified, and the number of colonies, frequency of closure, and diversity (H') were counted so that the Index of atmospheric purity (IAP) value at each observation location could be known. As many as 24 species from 14 lichen families with a total of 256 colonies were found in the four observation sites with the percentage of thallus crustose (62%), foliose (37%), and fruticose (1%). The highest lichen diversity was found in the CC area (2.62), followed by JD (1.99), PD (1.63), and the lowest in KK (0.90). The lowest IAP result was in KK (10.21), followed by PD (17.70) and JD (31.85). The location with the highest IAP was obtained at CC (46.65), indicating that the environmental conditions were still good, while other locations were polluted.

Keywords: *Biomonitoring, lichen, bioindicator, air quality*

1. Introduction

In the last few decades, there has been a decrease in air quality caused by several factors, including the use of fuel in transportation and motorized vehicles [1]. The higher the population growth in an area, the more transportation needs will increase [2]. Bandung area is densely populated area, with the growth of the transportation sector rising by 11% peryear. The ratio of the number of vehicles to the population is 3:4, with 72% of all registered cars being motorcycles [3]. This can increase air pollution due to toxic emissions suchas particulate matter (SPM10), lead, CO, hydrocarbons (HC), SOx, Ox, and NOx that are released into the environment, which results in decreased air quality and impacts public health [4]. Therefore, the government has issued an air pollution control policy to monitor ambient air quality using continuously operating devices, and the data can be observed directly. The installation devices process requires more costs and regular maintenance; therefore, its use isstill limited. Another approach as monitoring systems using physicochemical methods also limited to monitoring certain chemical compounds or pollutants and could not describe the effects of pollutants on living organisms [5]. Evaluating dynamic changes in environmental quality by observing at the response of living things systematically is a biomonitoring activity that uses the principle of repeated measurements on chemical/biochemical markers related to specific exposures to the observed bioindicators [6]. Bioindicators can reflect the quality of an environment or provide an overview of the ecological situation through its presence, absence, or behaviour closely related to a specific environmental status [7]. One of the organisms that can be used as a bioindicator is lichen. Lichen is a symbiotic organism between fungi (mycobiont) and algae (photobiont). The mycobiont component strengthens the body and absorbs water and minerals, while the photobiont

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Figure 1. Research Study Area

produces food by photosynthesis [8]. The interaction of these two organisms forms a unique morphology, reproduction,and classification system. Lichen is the most common organism used as an air pollution biomonitoring tool becauseit is proven effective in describing the conditions of various pollutants in the atmosphere. Lichens does not have a cuticle layer, stomata, and absorptive organs, therefore it passively absorbs water, air, and environmental nutrients into its intracellular tissues [3]. These characteristics make lichen susceptible to toxic or harmful compounds in the form ofair pollutants. In tropical countries, it has been demonstrated that environmental factors and air pollution directly affect lichen diversity and distribution [6]. Studies on lichen as a bioindicator of air quality have been carried out in various cities in Indonesia, such as Jakarta, Semarang, Pekanbaru, Kendari, and Medan. Among them is the discovery of the Lepraria incana species, a lichen species that can survive at lowto moderate air pollution intensities in Bandung [9]. Lichens respond to environmental changes by reflecting in their diversity, abundance, morphology, physiology, and pollutant accumulation in their thallus [6]. Therefore, biomonitoring ofair pollution using lichen could be based on two approaches, namely analysing the diversity of lichen and determiningheavy metals concentration in lichen thallus in areas with different levels of pollution [10]. Thus, this study utilizes these two biomonitoring approaches to monitor air qualityin four regions of Bandung Raya. This approach was carried out by analysing lichen diversity and abundance analysis to analyse lichen response to air quality in Bandung Raya and calculate the Index of Atmospheric Purity (IAP) based onthe frequency of thallus lichen closure. IAP value calculation helps determine air quality in Bandung Raya. According to [11], IAP is a quantitative analysis used to evaluate the level of pollution affecting lichens. This research was conducted in January-September 2022 in Bandung Raya area. Two locations are in the Bandung city, namely Ir. Haji Djuanda street (JD) (6°52’22”S;107°36’59”E) and Kebon kawung street (KK) (6°54’45”S;107°36’05”E), and two locations in West Bandung Regency, namely Padalarangstreet (PD) (6°50’38”S;107°29’11”E), and Curug Cimahi (CC) (6°47’55”S;107°34’32”E) as comparison stations (Figure 1).

2. Methodology

2.1 Data collection

Lichen samples were taken purposively at each station. 10 sample trees were chosen with a minimum distance of 3 m between trees to avoid tree shade. The area of lichen observation was carried out on the surface of the bark on the side facing the road as high as ±130 cm from the ground surface, using a transparent plastic quadrant frame measuring 25x25 cm [12]. After installing the plastic quadrant frame on the tree trunk, the type and amount of lichen in the quadrants were recorded. Lichen samples were taken by scraping the bark using a knife, then stored in an envelope and labeled/ described in the form of name, date, and place of sampling, as well as color and life form of lichen for further analysis. The coordinates of sampling station were recorded using GPS.

Environmental data collection

The environmental factors measured were air temperature and humidity, light intensity, tree bark pH, tree distance to the main road, and the volume of vehicles passing through the observation area. Air temperature and humidity measured using a sling psychrometer while for light intensity, using a lux meter. Microclimate data collection was carried out with three repetitions starts at 09.00 – 11.00 at each station. pH measurements on tree bark were carried out based on the method [13] with modifications. Research [2] suggests that the method used for calculating traffic volume is carried out at peak hours at The traffic volume calculated only motorized vehicles at peak hours suggested by (2) that is 07.00-08.00 WIB or 16.00-18.00 WIB. Measuring the distance of trees to

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the road is done using a rolling meter.

2.2 Data Analysis

Lichen Diversity

The lichen species were identified by observing the morphology and anatomy of the lichen [14]. Then the diversity index from each observation station was calculated by the Shanon-Weiner (H') diversity index with the formula:

$$H' = \sum_{i=1}^{s} (pi)(ln\ pi\ )$$

H' = Shannon-Wiener Diversity Index
Pi = ∑ni/ N
Ni = Number of individuals (species)
N = Number of individuals of all species

The calculation of lichen cover area is calculated based on the formula:

$$A = \frac{Wt}{Wi}\ x\ 1\ cm^2$$

A = Area of lichen closure
Wt = Total weight of HVS paper measured by area (mg)
Wi = Weight of HVS paper with an area of 1 cm^2

The lichen cover area is expressed in %, the closing percentage is calculated based on the formula:

$$\text{Frequency } (\%) = \frac{A}{A_k} x\ 100\%$$

A = Covering an area of lichen
Ak = Area squared (25 cm x 25 cm)

Calculation of the value of IAP (Index of Atmospheric Purity)

Environmental quality assessed by Index of Atmospheric Purity (IAP)[13]:

$$IAP = \frac{1}{10} \sum_{i}^{n} (Qi\ x\ fi)$$

IAP = Index of Atmospheric Purity
Qi = Ecological index (average number of species i), calculated based on the number of species found in an area divided by the total number of species found in the entire observation area.
Fi = Index of combination between frequency and lichen cover in an area

The meaning of the IAP values will be represented by table 1 [12]

Table 1. Interpretation of the IAP (Index of Atmospheric Purity) values

Pollution level	Criteria	Description
Level A	0≥IAP≤12,5	Very high pollution
Level B	12,5<IAP≤25	High pollution
Level C	25<IAP≤37,5	Moderate pollution
Level D	37,5<IAP≤50	Low pollution
Level E	IAP>50	Very low pollution

Statistic Analysis

The relationship between environmental parameters such as humidity, light intensity, temperature, tree bark pH, and tree distance from emission sources (roads) with lichen cover frequency analyzed using Spearman's correlation and factor analysis using Principal Component Analysis (PCA). This analysis is one of the mathematical models to describe the relationship between air quality and microclimatic factors that can be used to understand how changes in microclimatic. The data analyzed statistically using *Minitab 21*.

3. Results and Discussion

3.1. The proportion of lichen species based on morphology

Identification results showed that 24 species from 15 lichen families grew on tree substrates at observation stations in Bandung Raya. The number of lichens found at each stationis shown in Table 2. Lichens in Bandung Raya have varying colours, shapes, and thallus sizes. The lichen thallus colours included orange, green, grey, white, and yellow, with a talus size of ± 4-40 cm2. The most common lichens were found at observation stations CC as controls, JD, and PD, and the least was found in KK. The lichen species found were from different families, and based on the morphology of the thallus, the lichens were grouped into crustose (62%), foliose (37%), and fruticose (1%) (Figure 2).

In this study, the lichens analysed were lichens attached to tree trunks with a circumference of ≥50 cm (Table 3). Each tree species has a different canopy and bark texture, so lichens also has preference living and growing on certain tree species. Lichen cannot be found in all types of trees because, according to research conducted by [13], differences in tree bark texture

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Table 2. The total number of lichen species

Lichen Species	Family	Morphological Type	∑ Colony
Cryptothecia striata	Arthoniaceae	Crustose	26
Bacidia viridifarinosa	Bacidiaceae	Crustose	17
Dirinaria applanate	Caliciaceae	Foliose	4
Dirinaria picta	Caliciaceae	Foliose	18
Buellia sp.	Caliciaceae	Crustose	5
Chrysothrix xanthina	Chrysotrichaceae	Crustose	3
Leptogium sp.	Collemataceae	Foliose	5
Collema subflaccidum	Collemataceae	Foliose	6
Graphis sp.	Graphidaceae	Crustose	17
Hemithecium chrysenteron	Graphidaceae	Crustose	4
Lecidella elaeochroma	Lecanoraceae	Crustose	7
Lecanora sp.	Lecanoraceae	Crustose	14
Megalospora tuberculosa	Megalosporaceae	Crustose	4
Parmelia sp.	Parmeliaceae	Foliose	22
Parmothrema sp.	Parmeliaceae	Foliose	16
Heterodermia japonica	Parmeliaceae	Foliose	4
Canoparmelia aptata	Parmeliaceae	Foliose	5
Phlyctis argena	Phlyctidaceae	Crustose	23
Fulgensia sp.	Phlyctidaceae	Crustose	17
Physcia sp.	Physciaceae	Foliose	12
Pyxine cocoes	Physciaceae	Foliose	15
Lepraria incana	Stereocaulaceae	Crustose	50
Xanthoria sp.	Teloschistaceae	Foliose	3
Ramalina sp.	Ramalinaceae	Fruticose	4

Table 3. Lichen-growing substrate tree

Tree Species	Research Area			
	JD	KK	PD	CC
Artocarpus altilis	√	√	√	√
Swietenia mahagoni	√	–	–	–
Filicium decipiens	–	√	√	–
Roystonea regia	√	–	√	–
Mimusops elengi	–	–	√	–
Ficus benjamina	–	√	–	–
Maesopsis eminii	–	–	√	√
Schima wallichii	√	–	–	–
Toona sureni	–	–	–	√
Eucalyptus deglupta	–	–	–	√
Pterocarpus indicus	–	√	–	–
Samanea saman	√	√	√	–
Diospyros celebica	–	–	–	√

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Figure 2. Crustose thallus lichen: (a) *Lepraria incana*, (b) *Phlyctis argena*, (c) *Chrysothrix xanthina*, (d) *Lecidella elaeochroma*, (e) *Fulgensia* sp., (f) *Lecanora* sp., (g) *Graphis* sp., (h) *Buellia* sp., (i) *Hemithecium chrysenteron*, (j) *Cryptothecia striata*, (k) *Megalospora tuberculosa*, (l) *Bacidia viridifarinosa*

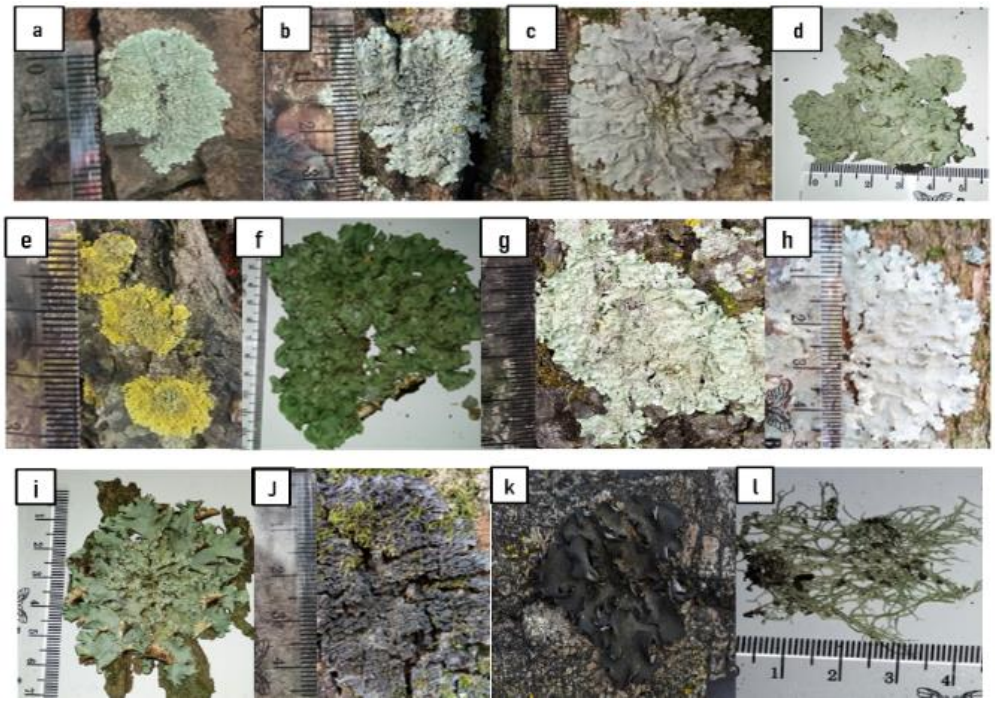

Figure 3. Foliose and fruticose thallus lichen: (a) *Dirinaria applanata*, (b) *Dirinaria picta*, (c) *Pyxine cocoes*, (d) *Parmelia* sp., (e) *Xanthoria* sp., (f) *Parmotrema* sp., (g) *Canoparmelia aptata*, (h) *Physcia* sp., (i) *Heterodermiajaponica*, (j) *Collema subflaccidum* (k) *Leptogium* sp. (l) *Ramalina* sp.

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affect lichen growth regardless of the various pollutant factors exist around the study site. Differences in the type and texture of tree bark as a lichen substrate affect the type of lichen that can grow.

Lichens in Bandung Raya have varying in colours, shapes, and thallus sizes. The lichen thallus colours included orange, green, grey, white, and yellow with a thallus size of ± 4-40 cm^2. The highest number of lichens were found at the CC observation station as a control, following by station JD,PD, and the least in KK. Based on the morphology of the thallus, the crustose type has a higher number and found in allobserved tree species in Bandung Raya site. Crustose lichen isa type of lichen that is strongly attached within a colony shapeto be rounded although sometimes has an irregular shape. The variation of lichen with a basic crustose form can be seen in figure 2.

Lichen foliose is a type of lichen with a thallus shape like a leaf blade. This type of lichen is commonly found in the CC area, and lichen fruticose is member of Ramalinaceae witha light green thallus. This species is often seen hanging from the bark of tree trunks at an altitude of > 1.2 meters from the ground. Research by [15] shows that this species grows in an open areas with low levels of air pollution, therefore, this species could also indicate of air pollution (Figure 3).

Number of Colonies and Frequency of Lichen Closure

Based on the number of colonies, species *L. incana*, *D. picta*, and *P. cocoes* had colonies that could be found in all observation sites. Meanwhile, *Ramalina* sp., *H. japonica*, *M. tuberculosa*, *H. chrysenteron*, and *D. applanate* were only found at one observation area and had fewer colonies than other species. The presence and absence of lichen species in an area indicate the potential of these species to become bioindicators of environmental quality. At each observation station, several lichen species have a higher frequency of closure than other species, related to the tolerance range of lichens to survive in various air pollution conditions and surrounding environmental conditions. Based on figure 4, it can be seen that the species in the KK and PD locations have fewer species than in JD and CC locations. Species in urban areas such as KK and PD areas are generally tolerant of air pollution. In general, susceptible species in this area experience an increase in frequency as air pollution increases.

The total frequency of lichen cover is much influenced by environmental factors such as humidity, light intensity, temperature [12], pH of tree bark, and tree distance to emission sources (roads), where the higher frequency of lichen cover indicates its ability to survive in the surrounding environment. In addition, lichen species are more chemically protected to

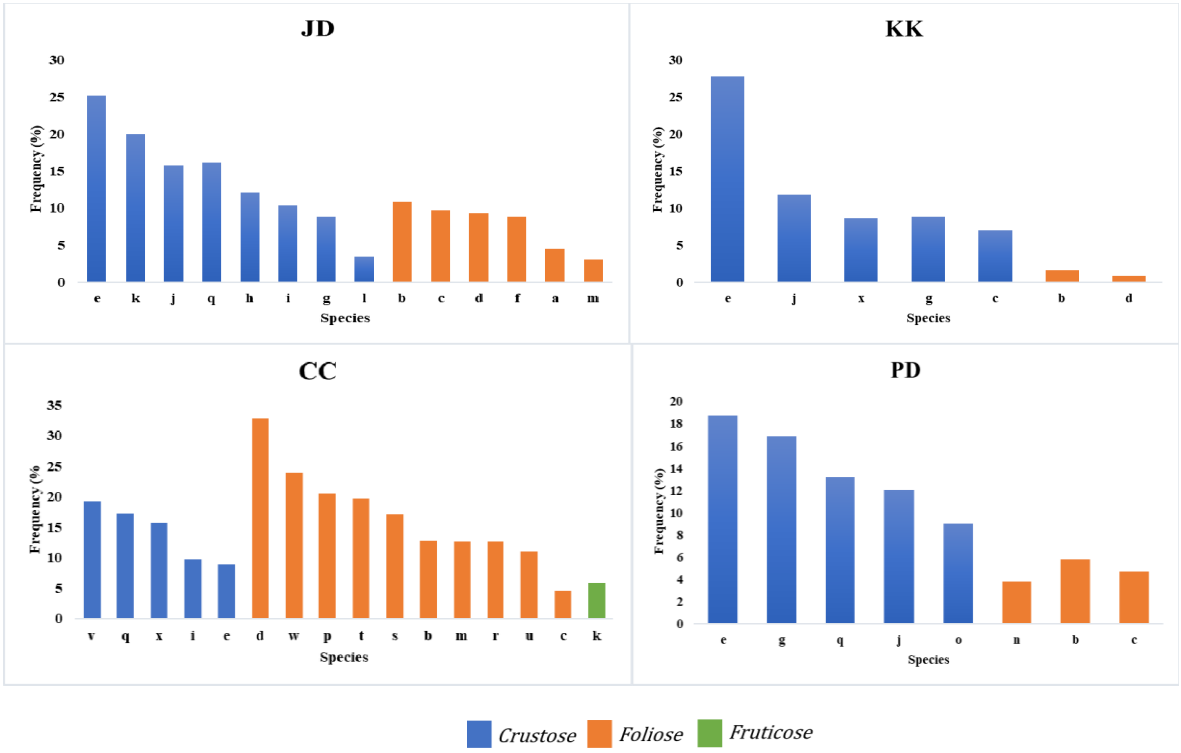

Figure 4. Frequency of lichen cover in all observation areas: (a) *Dirinaria applanata*, (b) *Dirinaria picta*, (c) *Pyxine cocoes*, (d) *Parmelia* sp., (e) *Lepraria incana*, (f) *Xanthoria* sp., (g) *Phlyctis argena*, (h) *Chrysothrix xanthina*, (i) *Lecidella elaeochroma*, (j) *Fulgensia* sp., (k) *Lecanora* sp., (l) *Graphis* sp., (m) *Collema subflaccidum*, (n) *Buellia* sp.,(o) *Hemithecium chrysenteron*, (p) *Parmotrema* sp., (q) *Cryptothecia striata* (r) *Ramalina* sp., (s) *Leptogium* sp., (t) *Canoparmelia aptata*, (u) *Physcia* sp., (v) *Megalospora tuberculosa*, (w) *Heterodermia japonica*, (x) *Bacidia viridifarinosa*

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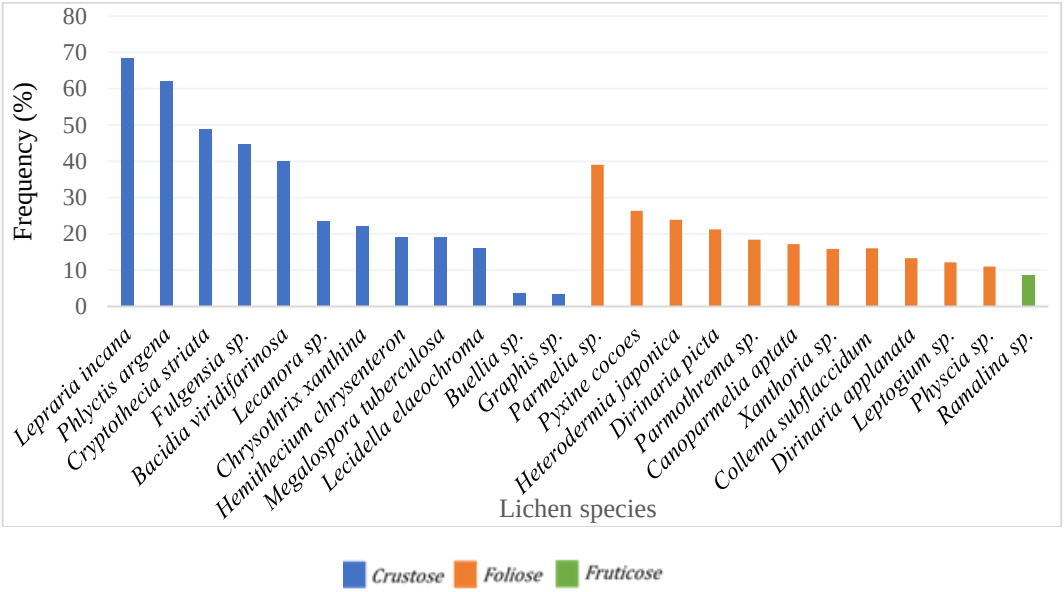

Figure 5. The total frequency of lichen cover

live in urban areas with higher pollution levels, drier, and more light intensity [12]. Figure 5 shows that there are three lichen species with the highest total coverage frequency for the crustose type, namely *L. incana* (68%), *P. argena* (62%), and *C. striata* (49%). In contrast, the foliose type is *P. cocoes* (39%), *Parmelia* sp. (26%), *D. Picta* (24%). This shows that the six lichen species were most often found in the observed trees. This indicates that the tolerance range of the six lichen species is large enough to grow in various environmental conditions. Lichens that are rarely found at observation stations are shown to have low-frequency values, including *Ramalina* sp. (0.09%), *Graphis* sp. (0.04%), and *Buellia* sp. (0.03%).

3.2. Correlation of lichen presence on microclimate factors, substrates, anthropogenic

The environmental conditions of an area can affect the diversity of lichens in that area. In this study, Principal Component Analysis (PCA) is a statistical technique that can be used to reduce the dimensionality of data while preserving as much variability as possible. In the context of studying the presence of lichens, PCA can help identify the most important factors (variables) influencing lichen distribution, such as microclimate conditions, substrates, and anthropogenic influences. According to [3], lichen species richness is affected by the humidity, light intensity, and temperature of an area which is indicated by lower lichen species richness in drier places. To determine the correlation between the frequency of lichen cover and environmental factors consisting of average humidity, light intensity, temperature, pH of tree bark, and tree distance to emission sources (roads), the Spearman correlation statistical test was carried out (Table 4). The results show that humidity, tree bark pH, and tree distance to anthropogenic sources (roads) strongly correlate with lichen covering frequency. Humid air conditions indicate an increase in the frequency of lichen closure. The neutral pH of the substrate supports this because the acidity of the substrate is also affected by the distance of the substrate from anthropogenic sources. Meanwhile,light intensity, temperature, and vehicle volume negatively correlate with the frequence of thallus lichen closure. High light intensity will increase air temperature, decreasing the

Table 4. Correlation of lichen presence on microclimate factors**,**

Variable	Temperature	Light intensity	Distance	Vehicle volume	pH of tree bark	Humidity	Fr (%)
Temperature	1						
Light intensity	0.84**	1					
Distance	-0.614**	-0.768**	1				
Vehicle volume	0.660**	0.758**	-0.918**	1			
pH of tree bark	-0.489*	-0.625**	0.862**	-0.763**	1		
Humidity	-0.656*	-0.580	0.452	0.562	0.514*	1	
Fr (%)	-0.489*	-0.625**	0.862**	-0.763**	0.718**	0.614**	1

** Correlation is significant at the 0.01 level (2-tailed).
** Correlation is significant at the 0.05 level (2-tailed).

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frequency of thallus lichen closure [11].

The PCA results presented in Figure 6 show that *Lepraria incana*, *Phlyctis argaena*, *Fulgensia* sp., are in quadrant IV, together with *Ficus benjamina* and *Pterocarpus indicus*. Thevariables that characterise this quadrant are temperature andlight intensity. The three species are lichens identical to urbanareas with higher temperatures and light intensity, such as theenvironmental conditions in the KK area. In addition, researchby [16] showed that lichens that are tolerant to air pollution,such as *Lepraria* sp. and *P. Argaena*, will increase in the amountand frequence of thallus lichen closure along with increasingtemperature and light intensity with high transport activity. Asin the Philippines, *Lepraria* sp. predominates in cities close to intense transport circulation This happens because the talus can accumulate elements such as heavy metals up to 97% [17].*Graphis* sp., *D. applanate*, *Parmelia* sp., *Lecanora* sp., *Xanthoria* sp., *Buellia* sp., *Physcia* sp., *B. viridifarinosa*, *M.tuberculosa*, *H. chrysteron*, *C. striata*, *L. elaeochroma* species are in quadrant II, together with *Swietenia mahagoni*, *Schimawallichi*i, and *Samanea saman*. Tree-to-road distance and tree bark pH characterised this quadrant. It is suspected that the 12species can be found in tree substrates which are more acidicand close to roads such as the JD area. Research conducted by

[3] stated that species that are close to highways found more acidic substrates have the potential to neutralise heavy metals in the air.

In quadrant I, *C. sublaccidum*, *C. aptata*, *H. japonica*, *Leptogium* sp., *Ramalina* sp., and *Parmothrema* sp. were found along with *Eucalyptus deglupta*, *Diospyros celebica*, *Toona sureni*. The variable that characterises this quadrant is air humidity. The presence of these species predominates in shady areas with high air humidity, such as the CC area. On the other hand, fruticose lichens, like *Ramalina* sp. found in quadrant I, namely lichen species is affected by moisture. This occurs because *Ramalina* sp. could not be found in all observation stations. After all, this group usually lives in moreundisturbed areas with higher moisture levels and a non-acidicbark pH [13]. Findings of several species, such as *Ramalina* sp. and *Leptogium* sp., which are only found in the CC area, shows that the area still has good air quality because this lichen is sensitive to acidification by air pollution [4]. CC hasmore closed-canopy trees than other areas, making it easier fororganisms to grow. In Mediterranean forests, the growth and development of lichens are closely linked to environmental conditions such as low human disturbance, high shrub cover, and areas with steeper slopes. These factors contribute to creating more stable microclimates and lower levels of humanactivity, which are favorable for lichen communities. Forexample, lichen diversity tends to be higher in forests with dense canopies and lower human impact, as seen in certain oakforests in Spain. Shaded north-facing slopes and areas with reduced disturbance provide optimal conditions for epiphytic

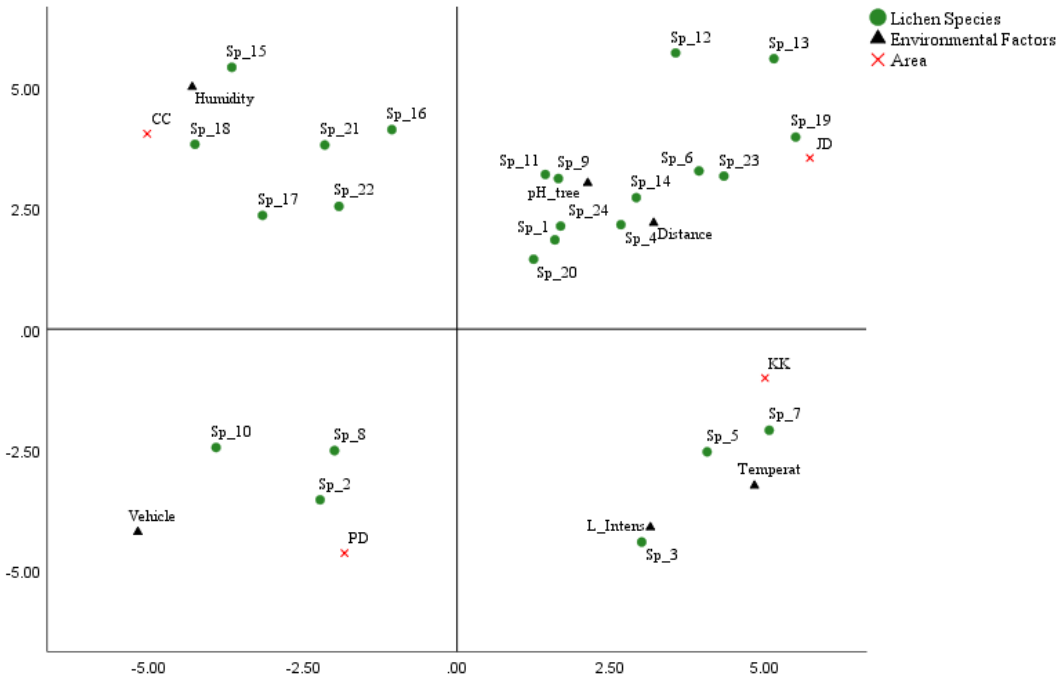

Figure 6. PCA results for lichen species data. **Quadrant I (CC Area)** consists of Sp 15: C. *sublaccidum*, Sp 16: C. *aptata*, Sp 17: H. *japonica*, Sp_18: *Leptogium* sp., Sp 21: *Ramalina* sp., Sp 22: *Parmothrema* sp.; **Quadrant II (JD Area)** consists of Sp 1 *Graphis* sp., Sp 4: D. *applanate*, Sp 6: *Parmelia* sp., Sp 9: *Lecanora* sp., Sp 11: *Xanthoria* sp., Sp 12: *Buellia* sp., Sp 13: *Physcia* sp., Sp 14: B. *viridifarinosa*., Sp 19: M. *tuberculosa*, Sp 20: H. *chrysteron*., Sp 23: C. *striata*., Sp 24: L. *elaeochroma*.; **Quadrant III (PD Area)** consist of Sp 2: P. *cocoes*, Sp 8: L. *elaeochroma*, Sp 10: H. *chrysenteron*; **Quadrant IV (KK Area)** consist of Sp 3: L. *incana*, Sp 5: P. *argaena*, Sp 7: *Fulgensia* sp.

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lichens, allowing them to thrive due to higher air humidityand less exposure to direct sunlight [18]. In temperate forestsof India, *Quercus semecarpifolia* trees in open canopy forests exhibit the highest lichen cover, with figures reaching up to 70%, while trees in closed canopy forests show only about 40% lichen cover. This variation in lichen cover is influenced by several factors, including tree canopy openness, tree size, and the physical properties of the tree bark [19].

In quadrant IV, *C. xanthina, P. cocoes* and *D. picta* were found together with *Roystonea regia, Artocarpus altilis*, and *Filicium decipiens*. The variable that characterises this quadrant is the volume of vehicles. .these three species allow it to live in areas with high traffic volumes. Species *L. incana, P. cocoes, P. argaena, Fulgensia* sp., *C. xanthina*, and *D. picta* have the potential as bioindicators of medium-high air pollution because these species are lichens whose existence is found close to anthropogenic sources with high traffic density like in KK, PD, and JD.

the species diversity index value in KK and PD is included in criterion H'<1 namely low lichen species diversity. Based on the dominance index at JD and CC observation stations, the dominance of lichen species is low because there are no dominant lichen species in the area, while in PD and KK, the dominance of lichen species is moderate because there are several lichen species that dominate in the area.

Understanding an organism's pattern of diversity and dominance is critical in conservation and management. In recent years, the design of lichen diversity in Bandung Raya shows that the index value of lichen species tends to decrease along with poor air quality. Ecological factors are essential in lichen species' growth, development, distribution, and diversity. Variations in microclimate conditions, incredibly light, water, and nutrient intensity, driven by local disturbance sources such as roads or agriculture, different land uses, or habitat fragmentation, can affect lichen diversity. A study by [19] stated that metal concentrations accumulated in lichens

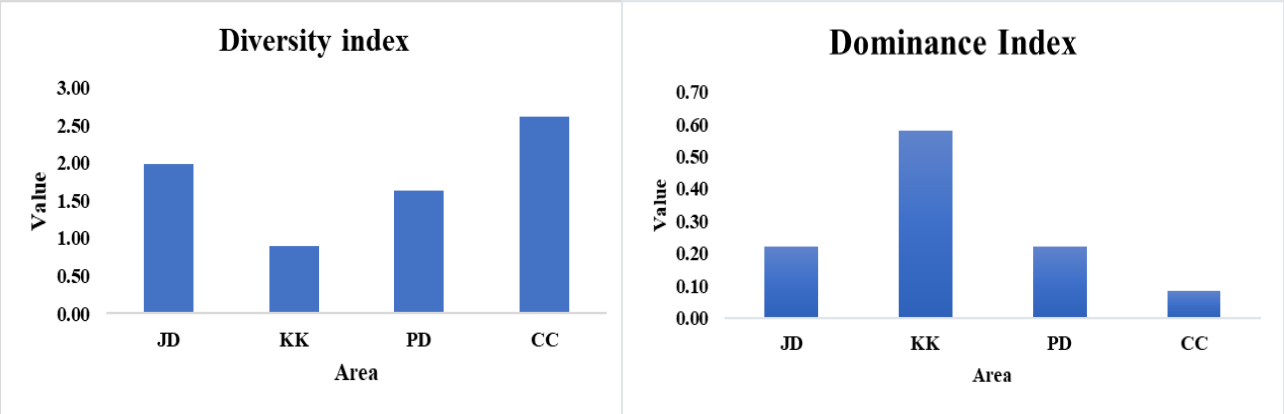

Figure 7. Diversity and dominance Index of lichen

Table 5. Index of Atmospheric Purity measurement results

Area	IAP	Pollution level	Description
Djuanda street	24,85	Level B	High pollution
Kebon Kawung street	10,21	Level A	Very high pollution
Padalarang street	17,70	Level B	High pollution
Curug Cimahi	46,65	Level E	Very low pollution

3.3. Diversity Index and Lichen Dominance

Diversity and lichen dominance can be taken as an estimate of air quality. The higher the diversity value and the lower the dominance value indicate good environmental conditions and vice [17]. In figure 7, the index value of lichen species diversity in CC is included in the index criteria H'>3, which indicates very abundant lichen diversity, JD has an index value of 1<H'<3 which is great lichen diversity, while

correlated with distance to the source of pollution, climatic factors, and land cover, significantly impacting lichen diversity. One of the primary markers of anthropogenic pollution on earth is the decrease in lichen diversity demonstrated at the end of the 19th century in Paris.

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3.4. Index of Atmospheric Purity

According to the categorization of air quality levels proposed by [3] ba sed on the IAP value, the observation stations with the highest to lowest levels of air pollution are KK, PD, JD, and CC (Table 5). The four observation stations show differences in vehicle emissions caused by differences in traffic volume and good air environmental conditions so that living organisms such as lichens can be maintained. This is to research conducted by [18] that highly disturbed city sites (urban areas with high disturbance) fall into the same category, namely levels A and B. In addition to the high traffic intensity and density that causes air pollution, urban areas also have limited availability of substrates, resulting in changes in microhabitat conditions that can affect biodiversity and lichen frequency. Lichen biodiversity and frequency will generally increase as the distance from the emission source are further away, and the IAP value will increase with the distance from urban areas. The distinguishing factor of the IAP value at these four observation stations is the difference in land use. According to [3] IAP in industrial areas, toll roads, and other urban areas tend to be lower than in areas with natural environmental conditions. Apart from the CC area, the other three regions have the same land use, so it is also observed that the IAP value reflects the similarity of lichen variety and closing frequency.

4. Conclusion

The objectives of research on lichens as air quality indicators are to understand and evaluate their ability to detect and monitor air pollution levels. Study different lichenspecies and their distribution across various environments to determine their sensitivity to air pollutants. A total of 24 species from 14 families with a total of 256 lichen colonies were found at the observation station. Based on their thallus morphology, (62%) percent lichen were grouped into crustose type (62%), foliose type (37%), and fruticose type (1%). Lichen species that were high in frequency in moderate to very high levels of pollution were *Lepraria incana*, *Pyxine cocoes*, *Phlyctis argaena*, *Fulgensia* sp., *Chrysothrix xanthina*, and *Dirinaria picta*. Those six species are identical to urban areas with higher temperatures and light intensity, such as environmental conditions in the KK area. On the other hand, the lichen *Ramalina* sp. is a species sensitive to air pollution, so it can only live in low pollution levels, such as areas in CC because this lichen is sensitive to acidification by air pollution. CC has more closed-canopy trees than other areas, making it easier for organisms to grow. The highest lichen diversity was found in the CC area (2.62), followed by JD (1.99), PD (1.63), and the lowest in KK (0.90). The lowest IAP result was in KK (10.21), followed by PD (17.70) and JD (31.85). The location with the highest IAP was obtained at CC (46.65), indicating that the environmental conditions were still good, while other locations were polluted. Environmental factors like humidity, tree bark pH, and tree distance to anthropogenic sources (roads) are strongly correlated with the frequency of lichen closing, while light intensity, temperature, and vehicle volume are negatively correlated with the frequency of thallus lichen closure.category, namely levels A and B. In addition to the high traffic intensity and density that causes air pollution, urban areas also have limited availability of substrates, resulting in changes in microhabitat conditions that can affect biodiversity and lichen frequency. Lichen biodiversity and frequency will generally increase as the distance from the emission source are further away, and the IAP value will increase with the distance from urban areas. The distinguishing factor of the IAP value at these four observation stations is the difference in land use. According to [3] IAP in industrial areas, toll roads, and other urban areas tend to be lower than in areas with natural environmental conditions. Apart from the CC area, the other three regions have the same land use, so it is also observed that the IAP value reflects the similarity of lichen variety and closing frequency.

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Application of Compost Tea and Ascorbic Acid to Increase Productivity and Antioxidant Activity of The Common Bean (*Phaseolus vulgaris* L.)

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Abstract

The common bean is a functional food rich in nutrients and bioactive compounds that support good health. Improving common bean yield is essential for enhancing nutrition within communities. This study examined the effects of compost tea, ascorbic acid, and their combination on the productivity and antioxidant activity of upright common beans. The experiment involved six treatments, including a control (water spraying) (P1), ascorbic acid (P2), manure compost tea (P3), BSF larvae production residue (BSFR) compost tea (P4), manure compost tea and ascorbic acid (P5), and BSFR compost tea and ascorbic acid (P6). All treatments, except P6, showed significant differences in productivity. P2 recorded the highest yield at 8,13 tons/ha/cycle and increased productivity by 19,03%, followed by P5 (9,22%) and P3 (3,95%) compared to P1 (control). P4 had a slight reduction in productivity by 3,95% than P1. A combination of compost tea and ascorbic acid increased productivity by 5,07% for P5 and 4,63% for P6 compared to the treatment without combination with ascorbic acid. P2 had the lowest IC50 value, 17,46 µg/ml, indicating the highest antioxidant activity. Compost tea and ascorbic acid combined reduced the IC50 value by 23,12% for P5, but P6 increased it by 32,59%. P5 improved the antioxidant activity of common bean pods when combined with compost tea and ascorbic acid, while P6 demonstrated a better effect without the combination. P3 and P6 had higher IC50 values than the control but lacked the potential to improve the antioxidant activity of common bean pods.

Keywords: *antioxidant; ascorbic acid; compost tea; productivity; Phaseolus vulgaris*

1. Introduction

Common beans (*Phaseolus vulgaris* L., hereafter simply abbreviated as *P. vulgaris*) is a plant that offers various benefits to the human body. They are known to contain 34 calories, 7,2 g carbohydrates, 2,4 g protein, 0,3 g fat, 1,9 g fiber, 42 mg phosphorus, 101 mg calcium, 0,7 mg iron, 250 mg potassium, 8 mg sodium, 11 mg vitamin C, and 89,6 g water in every 100 grams [1]. Consumption of common bean pods is also known to reduce the risk of obesity and diabetes, control diabetes, reduce the risk of coronary heart disease and colon cancer, act as a source of dietary fiber, improve gastrointestinal response, prolong the existence of lipoproteins to reduce bad cholesterol levels in the body and prevent breast cancer [2].

Common beans are a vegetable commodity that requires production development. High common bean production can align with the improvement of nutrition in the community. Common beans are classified as nutritious functional foods because they contain various bioactive compounds that benefit health. These bioactive compounds include polyphenols, anthocyanins, condensed tannins, and flavonoids in the form of quercetin, providing high antioxidant capacity [3]. One of the efforts that can be made in developing common bean production is by increasing its productivity to broaden its economic prospects and attract farmers to plant common beans.

Efforts to boost common bean productivity include the application of ascorbic acid. Research shows that spraying ascorbic acid concentrations of 200 mg/L and 400 mg/L on the plant's shoot can increase productivity, antioxidant activity, and secondary metabolites under water-stress conditions [4]. Geeth and Abdel-Aziz also discovered that the application of 200 mg/L of ascorbic acid to common beans can enhance the chemical composition of leaves and pods, resulting in an increased yield [5]. Additionally, Hayat et al. found that the

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application of 1.5 mM ascorbic acid could improve common bean productivity under drought-stress conditions [6]. Another effort that can be made to increase common bean productivity is through the application of fertilizers, both synthetic and organic. Organic fertilizers are considered to have a more sustainable effect on the ecosystem compared to synthetic fertilizers. These fertilizers can be solids or liquids, including liquid organic fertilizers such as compost tea. Compost tea is made by soaking bags of solid fertilizer in water to obtain liquid extract rich in nutrients, organic compounds, and beneficial microbes [7]. Ibrahim et al. revealed that compost tea treatment of common beans could increase their productivity through the biostimulation effect provided by compost tea [8]. Another potential of compost tea has also been shown to suppress damping-off disease caused by *Rhizoctonia solani* on common bean plant sprouts [9]. Additionally, compost tea made from cow and chicken manure is also able to act as a disease-control agent in soybean plants [10].

This study aims to determine the effect of compost tea and ascorbic acid and the effectiveness of their combination on the productivity and antioxidant activity of upright common bean (*P. vulgaris*), building on previous explanations.

2. Methodology

2.1 Time and Place

The cultivation phase took place during January-March 2023 in Parongpong, West Bandung Region, West Java, specifically at the coordinates -6° 49’ 50.196” for latitude, and 107° 35’ 21.5946” for longitude. Laboratory examinations and data processing transpired at Engineering Laboratory 1A, within the Jatinangor Campus of Bandung Institute of Technology.

2.2 Research Design

The research was structured using a Randomized Complete Block Design (RCBD), encompassing six distinct treatments and four replications as delineated below. The seed variety used was GYPSY-1 upright common bean (*P. vulgaris*).

P1: Control (water spraying)

P2: Ascorbic acid (AsA)

P3: Manure compost tea (MCT)

P4: BSF larvae production residue / BSFR compost tea (BCT)

P5: Manure compost tea + ascorbic acid (MCT + AsA)

P6: BSFR + ascorbic acid (BCT + AsA).

2.3 Cultivation Procedure

The cultivation procedures adhered to the standard operational procedure (SOP) for common bean cultivation from The Ministry of Agriculture Indonesia [11]. This included the use of a drip irrigation system for watering, weeding at 30-40 days after sowing (DAS) to remove interfering weeds, and treatments applied through spraying on the plant shoot area/foliar spray using a hand sprayer. Treatments were administered every seven days, commencing at 17 DAS when the first trifoliate leaves had fully developed. The compost tea fertilizer was applied with a concentration of 50% (fertilizer: water = 1:1, v/v), while the ascorbic acid treatment utilized a concentration of 400 mg/L. Spraying was conducted to cover 70% of the plant shoot area, in the morning at 08:00 - 10:00 AM. Common beans were harvested three times after reaching 51 DAS, and harvest data were meticulously collected.

2.4 Compost Tea Preparation

Compost tea preparation involves placing solid fertilizer material into a gauze bag or other porous fabric to act as a filter. This bag was then immersed in a barrel filled with water and an aerator pump. The ratio of solid fertilizer to water was maintained at 1:4 v/v, with the addition of 2.5 ml of molasses. This mixture was left to soak for three days to dissolve the nutrients, while the aerator pump continuously agitated it and occasional stirring was performed. The successful compost tea solution was identified by its earthy scent and readiness for application.

2.5 Measurement and Data Collection

2.5.1 Plant Height, Leaf Count, and Leaf Area

Plant height was measured using a cloth meter, leaf count was manually recorded on fully developed leaves, and leaf area was assessed through digital image analysis. Leaves were photographed, and their area was calculated by measuring pixel units in the IMAGE-J application.

2.5.2 Yield and Productivity

Yield parameters included pod wet and dry weights, pod organic and ash content, number of pods, pod length, pod diameter, and weight per pod, all measured with an analytical balance. Dry weight was obtained by drying 100 g samples in an oven for 24 hours at 105°C. Organic and ash (mineral) content were determined via dry ashing. Initially, 0.5 g of dry sample in a crucible was placed in a furnace at 550-600°C for 3-4 hours. The results of ashing were then weighed with an analytical balance following this formula:

$$Ash\ Content = \frac{ash\ weight}{dry\ sample\ weight} \times 100\%$$

$$Organic\ Content = 100\% - ash\ content$$

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The values for each parameter were averaged based on the treatment provided. Productivity was calculated by multiplying the production per plant by the number of plants per hectare at a density of 9,6 planting holes/m^2 (referring to the research design). The number of plants per hectare was determined using a simplified approach based on a 10 m x 10 m plot, which was then extrapolated to a one-hectare land area. It was assumed that the 10 m x 10 m plot contained seven beds measuring 1 m x 10 m, with a spacing of 0,4 m between beds, resulting in a planted land area of 7.000 m^2. Productivity was computed using the formula:

$$\text{Productivity} = \text{pod weight per plant} \times \text{number of plants per } m^2 \times \text{planted area per hectare}$$

2.6 Antioxidant Activity Test

Antioxidant activity testing was performed using the DPPH (1,1-Diphenyl-2-Picryl Hydrazyl) method, following the procedure by Nugrahani et al. [12] with slight modifications. Methanol extract from common bean pods was used as the test material.

2.6.1 Methanol Extraction of the Test Sample

A fine powder of common bean pods weighing 25 grams was extracted with 250 ml of methanol solvent pro analysis (PA) by maceration for 48 hours. The maceration results were then vacuum distilled and concentrated with a rotary evaporator at 65°C and 426 millibar pressure until a thick extract was obtained.

2.6.2 Preparation of DPPH Solution

A DPPH 0.5 mM stock solution was prepared by weighing 20 mg of DPPH, which was then dissolved in 100 ml of methanol PA in a 500 ml dark bottle and stored at 2-8°C. The determination of DPPH absorbance was carried out by preparing a blank solution, adding 1 ml of DPPH to 4 ml of PA methanol solution, allowing it to sit for 30 minutes in a dark place, and then measuring the absorbance with a spectrophotometer at a wavelength of 517 nm.

2.6.3 Preparation of Test Solution

A secondary standard solution was prepared by dissolving common bean extract and methanol solvent with a precise weight ratio of extract (mg) to methanol (ml) at 1:1 proportion, and this solution was placed within a dark bottle. Subsequently, serial solutions were created through dilution of the secondary standard solution to attain concentrations of 10 ppm, 20 ppm, 40 ppm, 60 ppm, 80 ppm, and 100 ppm, each comprising 2 ml. Meanwhile, for comparative purposes, a solution of ascorbic acid (vitamin C) was prepared at various concentrations (10 ppm, 20 ppm, 30 ppm, 40 ppm, 50 ppm, and 60 ppm), each in the volume of 2 ml. In the testing process, each test solution was supplemented with 1 ml of DPPH and allowed to incubate in darkness for 30 minutes. Following this incubation, the absorbance of each solution series was meticulously measured utilizing a spectrophotometer, with a specific focus on the wavelength of 517 nm. Once the absorbance values were acquired, the antioxidant activity of the methanol extract of common bean was determined by evaluating the percentage inhibition of the extract against DPPH free radicals, employing the formula:

$$\%\ Inhibition = (1 - \frac{A_{sample}}{A_{blank}}) \times 100$$

Here:

A blank = absorbance of the blank uptake

A sample = absorbance of the sample uptake

Calculation of the IC_{50} value, indicating the concentration needed to reduce 50% of the DPPH radical activity, was accomplished through a linear regression equation y = a + bx, where the y coefficient denotes % inhibition and the x coefficient signifies the IC_{50} value.

2.7 Data Analysis

The acquired data underwent thorough analysis using IBM SPSS Statistics software. The chosen statistical test was a one-way ANOVA, conducted with a 95% confidence level and a significance threshold of $\alpha = 0,05$. In the event of significant outcomes, further analyses were undertaken employingthe Duncan Multiple Range Test (DMRT) to explore the differences among treatment groups.

3. Results and Discussion

3.1 Nutrient Content Analysis of Compost Tea

Laboratory analysis conducted by the Lembang Agro Chemistry Laboratory on liquid organic fertilizer, including manure compost tea (MCT) and BSFR compost tea (BCT), revealed that, in general, neither of these compost teas (CTs) met the established quality standards. While the pH levels of both CTs were within the acceptable range as per the standards, other parameters yielded conflicting results. These parameters include C-Organic, macronutrients N, P, and K, C/N ratio, as well as micronutrients Fe, Cu, and Zn (Table 1).

Several factors influence the quality of CT, includingthe ratio of solid compost to water, the type of compostused, and aeration, which supports the growth of beneficial microorganisms [14]. It is crucial to consider the maturity index of solid compost before using it for compost tea, taking into account parameters like C/N ratio, pH, and EC (Electric Conductivity) [15]. The low nutrient content observed in MCT

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and BCT can be attributed to the initial compost substrate's immaturity, resulting in limited nutrient solubility. Immature compost fails to fully decompose organic matter into inorganic forms, affecting CT quality. However, CT contains a wealth of beneficial microbes, including actinomycetes, oomycetes, yeasts, filamentous fungi, and bacteria, which are abundant and classified as Plant Growth Promoting Rhizobacteria (PGPR).

PGPR plays a significant role in enhancing plant growth by facilitating nutrient solubilization, the release of growth hormones and enzymes, and nitrogen fixation. Additionally, CT contains humic acid and growth regulators, and the combination of these components can effectively suppress plant diseases [14]. Previous research has explored the utility of CT as a pathogen inhibitor for legume plants, demonstrating its potential as a biological control agent against damping off disease and other soil-borne diseases, while also promoting crop growth in common bean [9].

3.2 Growth of Plant Height, Number of Leaves, and Leaf Area

Common bean exhibit various phenological phases throughout their life cycle, characterized by the development of distinct plant organs. These phenological phases include V1 emergence, V2 primary leaves, V3 first trifoliate leaf, V4 third trifoliate leaf, R5 preflowering, R6 flowering, R7 formation of pods, R8 filling of pods, and R9 maturation [16].

3.2.1 Plant Height

Plant height growth demonstrates a significantly different response, as indicated by the results of the DMRT statistical test ($p \leq 0,05$) conducted between 23 and 35 days after sowing (DAS). The most significant increase in height occurred with treatment P3 at the end of the V3, V4, and R5 phases, measuring 32,74 ± 2,92 cm, 48,71 ± 4,82 cm, and 51,41 ± 4,81 cm, respectively. Conversely, treatment P2 resulted in the lowest height at the end of phase V3, measuring 26,24 ± 2,33 cm, which was not significantly different from treatments P5 and P6. Meanwhile, at the end of phases V4 and R5, the lowest effect was observed with treatment P6, yielding an average height of 40,17 ± 3,45 cm and 43,16 ± 3,99 cm, respectively. This analysis indicates that treatment P3 was the most effective in promoting common bean plant height growth during each growth phase compared to control (P1). Conversely, the combination of MCT and BCT with AsA exhibited a lesser effect so the combination did not significantly enhance plant height growth and tended to interact antagonistically with each other (Table 2).

Beneficial microbes in compost tea play a crucial role in supporting plant height growth. According to Samet etal.'s research on bacteria in CT, they revealed that *Serratia liquefaciens* and *Achromobacter spanius* bacteria can act as plant growth-promoting rhizobacteria (PGPR) capable of producing the hormone auxin (indole acetic acid, IAA) [17]. Therefore, there is a tendency for the MCT treatment to be the

Table 1. Laboratory analysis results for manure compost tea and BSFR compost tea

No	Parameter	Units	MCT	BCT	Standard*
1	pH	-	7,1	6,75	4 - 9
2	C organic	%	0,26	0,13	Minimun 10
Macronutrient					
3	N total	%	0,06	0,05	% total NPK = 2 - 5
4	P_2O_5	%	0,07	0,02	
5	K_2O	%	0,14	0,08	
6	C/N	-	4,33	2,6	12-15**
7	Ca	%	0,02	0,02	-
8	Mg	%	0,01	0,002	-
Micronutrient					
9	Fe	ppm	14,2	0	90 – 900
10	Cu	ppm	6,6	0	25 – 500
11	Zn	ppm	3,6	1,2	25 – 500

*) Decree of the Minister of Agriculture of the Republic of Indonesia No 261/KPTS/SR.310/M/4/2019;
**) Mansyur et al. [13].

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Table 2. Plant height observations at 17 - 35 DAS.

Treatment	Day After Sowing (cm)						
	V3				V4		R5
	17	20	23	26	29	32	35
P1	12,09 ± 0,91 a	15,89 ± 1,33 a	21,78 ± 1,72 a	29,51 ± 2,89 ab	37,69 ± 3,50 a	46,07 ± 4,11 bc	49,46 ± 4,74 bc
P2	10,94 ± 0,47 a	15,23 ± 1,45 a	20,48 ± 1,75 a	26,24 ± 2,33 a	35,69 ± 3,53 a	43,51 ± 4,12 ab	46,79 ± 4,45 ab
P3	12,57 ± 1,03 a	16,57 ± 0,98 a	24,39 ± 1,96 b	32,74 ± 2,92 b	41,83 ± 4,03 b	48,71 ± 4,82 c	51,41 ± 4,81 c
P4	12,11 ± 1,19 a	16,79 ± 1,44 a	22,70 ± 1,86 ab	29,32 ± 2,40 ab	38,30 ± 3,35 a	44,14 ± 4,36 ab	47,36 ± 4,48 abc
P5	11,44 ± 0,53 a	15,32 ± 1,06 a	21,15 ± 1,70 a	27,63 ± 2,43 a	38,25 ± 3,65 a	43,04 ± 4,24 ab	46,19 ± 4,13 ab
P6	11,78 ± 0,74 a	15,55 ± 1,45 a	22,18 ± 1,57 ab	28,44 ± 2,76 a	35,14 ± 2,93 a	40,17 ± 3,45 a	43,16 ± 3,99 a

Notes: Numbers accompanied by the same letter in the same column show no significant difference in the 5% DMRT test.

most effective treatment for plant height growth parameters because it contains PGPR bacteria.

3.2.2 Number of Leaves

In general, the treatments administered had a significant impact on increasing the number of leaves, as indicated by the results of the DMRT statistical test ($p \leq 0,05$). Treatment P3 had the highest increase in leaf count, reaching 10,06 ± 0,63 leaves at the end of phase V3, which was significantly different from the other treatments. During phase V4, the highest leaf count increase was observed with treatment P5, at 21,13 ± 1,88 leaves, not significantly different from P3. Similarly, during phase R5, treatment P3 yielded the highest leaf count increase at 23,88 ± 2,05 leaves, significantly different from the other treatments. Conversely, the lowest leaf count growthresponse at the end of phases V3, V4, and R5 was recorded with treatment P6, at 7,63 ± 0,43 leaves, 17,94 ± 1,49 leaves (not significantly different from P1), and 20,19 ± 1,84 leaves, respectively. The analysis demonstrated that treatment P3 remained superior, while the P6 treatment showed an inverse effect. This suggests that the combination of AsA and BCT has not been effective due to a lower response, possiblydue to potential antagonistic interactions between these two components (Table 3).

The combination treatment of AsA and CT leads to excessive water accumulation in leaf organs, resulting in overly wet leaves that inhibit the evapotranspiration process. This condition induces stress in plants, causing wilting and, consequently, reduced plant growth yield [18]. A similar scenario can be observed in plant height growth, where data obtained from the combination of AsA and CT is notably lower, influenced not only by the antagonistic interaction between the two components. Other findings were reported in Ibrahim et al.'s research concerning the application ofCT on common beans. The application at varying levels had a substantial impact on the number of leaves per plantat various locations. Specifically, the application of 100 L/ha resulted in significantly higher leaf counts than the 50 L/ ha treatment and notably surpassed the control treatment [8]. The data suggested that as the dosage of compost tea fertilizer increased, there was a tendency for an enhanced response in terms of an increased number of leaves.

Table 3. Number of leaves observation at 17 - 35 DAS

Treatment	Day After Sowing (blade)						
	V3				V4		R5
	17	20	23	26	29	32	35
P1	1,44 ± 0,13 c	3,56 ± 0,13 ab	6,75 ± 0,50 a	9,00 ± 0,54 c	12,81 ± 0,97 ab	17,88 ± 0,66 a	20,44 ± 1,43 ab
P2	1,44 ± 0,13 c	3,25 ± 0,20 a	6,25 ± 0,35 a	7,94 ± 0,38 ab	11,50 ± 1,06 a	20,06 ± 1,51 ab	23,00 ± 2,07 abc
P3	1,81 ± 0,13 d	4,69 ± 0,24 c	7,69 ± 0,63 b	10,06 ± 0,63 d	14,88 ± 0,75 c	20,56 ± 1,72 b	23,88 ± 2,05 c
P4	2,19 ± 0,13 e	4,88 ± 0,32 c	7,88 ± 0,43 b	9,56 ± 0,55 cd	13,94 ± 1,14 bc	20,13 ± 1,65 ab	23,38 ± 1,93 bc
P5	1,25 ± 0,00 b	3,69 ± 0,24 b	6,69 ± 0,52 a	8,75 ± 0,61 bc	14,81 ± 1,40 c	21,13 ± 1,88 b	23,13 ± 1,74 abc
P6	0,81 ± 0,13 a	3,25 ± 0,29 a	6,25 ± 0,29 a	7,63 ± 0,43 a	12,38 ± 0,83 a	17,94 ± 1,49 a	20,19 ± 1,84 a

Notes: Numbers accompanied by the same letter in the same column show no significant difference in the 5% DMRT test.

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3.2.3 Leaf Area

The highest average leaf area response at the end of phase V3 was achieved with treatment P1, measuring 103.03 ± 9.37 cm², not significantly different from treatment P4. At the end of phases V4 and R5, the highest average response was observed with treatment P4, measuring 155.12 ± 15.10 cm² and 157.51 ± 14.94 cm², respectively, but not significantly different from P1. Overall, the application of MCT, BCT, AsA, and their combination did not yield a notably better effect on leaf area than the control (Table 4).

Leaf area growth is influenced by nutrients, the environment, and growth hormones. In general, the combination of CT treatment with separately applied AsA resulted in lower data analysis results compared to treatments without the combination. This may be attributed to the high concentration of AsA at 400 mg/L, which tends to disrupt the stability of the 50% concentration CT, rendering it less effective. AsA plays a crucial role as an antibacterial agent, and at certain concentrations, it can diminish the effectiveness of compost tea. AsA exhibits strong inhibitory effects against various microorganisms, including *Staphylococcus aureus*, *Enterococcus faecalis*, *Helicobacter pylori*, *Campylobacter jejuni*, *Mycobacterium tuberculosis*, and even fungi like Aspergillus [19]. Furthermore, it has been demonstrated to enhance the antibacterial activity of clindamycin, enabling it to inhibit *Streptococcus pneumoniae* [20]. AsA also serves as a sterilizing agent in explant preparation for tissue culture processes [21]. These attributes give rise to the potential for antagonistic interactions with CT.

Table 4. Leaf area observation at 17 - 35 DAS

Treatment	Day After Sowing (cm²)						
	V3				V4		R5
	17	20	23	26	29	32	35
P1	11,63 ± 2,98 a	27,18 ± 7,44 a	74,26 ± 3,35 b	103,03 ± 9,37 c	127,40 ± 7,36 c	153,97 ± 8,99 c	156,77 ± 8,44 c
P2	11,60 ± 3,12 a	36,32 ± 10,15 a	72,61 ± 3,43 b	98,01 ± 7,98 bc	120,28 ± 10,63 bc	144,12 ± 10,74 bc	146,04 ± 10,74 bc
P3	6,71 ± 1,94 a	24,79 ± 7,17 a	62,37 ± 4,35 a	89,94 ± 2,56 ab	117,18 ± 11,13 bc	144,58 ± 14,25 bc	147,17 ± 14,04 bc
P4	9,36 ± 2,38 a	32,98 ± 8,21 a	73,57 ± 6,36 b	102,74 ± 8,88 c	127,94 ± 10,16 c	155,12 ± 15,10 c	157,51 ± 14,94 c
P5	9,87 ± 2,52 a	34,36 ± 9,56 a	70,33 ± 3,43 b	95,67 ± 3,81 bc	113,08 ± 9,45 ab	134,46 ± 13,35 ab	136,68 ± 8,00 ab
P6	7,45 ± 2,14 a	24,82 ± 6,69 a	58,06 ± 4,08 a	82,18 ± 7,63 a	102,80 ± 9,45 a	125,17 ± 6,77 a	127,47 ± 12,22 a

Notes: Numbers accompanied by the same letter in the same column show no significant difference in the 5% DMRT test.

3.3 Yield

Yield parameters included wet and dry weights of pods, pod organic and ash content, number of pods, pod length, pod diameter, and weight per pod.

3.3.1 Pod wet and dry weights, organic and ash content

Treatment P2 yielded the highest average wet weight of common bean pods at 483,80 ± 39,043 grams, significantly different from the other treatments. Conversely, the lowest response was observed with treatment P4, producing pods with a wet weight of 373,05 ± 37,880 grams. Data analysis indicated that treatment P2 effectively increased pod wet weight production by 19,02% compared to treatment P1, followed by an increase of 9,2% with treatment P5, and 3,96% with treatment P3, while treatment P4 showed no significant difference from the control. Treatment P6 produced the lowest response, with wet weight production 3,89% lower than treatment P1. However, the response of common bean pod dry weight did not exhibit significant differences among treatments, suggesting that none of the treatments significantly increased pod dry weight yields (Table 5).

The application of AsA, in common beans serves essential functions such as maintaining several metabolic processes under stress conditions. AsA acts as a non-enzymatic antioxidant compound, serving as an electron donor to reduce the accumulation of reactive oxygen species (ROS) and as a substrate in enzymatic processes. The addition of 400 mg/L AsA has been demonstrated to increase the yield of common bean (*P. vulgaris*) [4]. As an antioxidant, AsA aids in enhancing plant growth and yield by acting as a secondary nutrient or micronutrient. AsA plays a pivotal role in regulating plant growth, including processes like cell elongation and division, and bolstering the plant's immune system against

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environmental stressors [22].

The analysis of the average organic content of common bean pods showed no significant differences between treatments, indicating that each treatment failed to increase the organic content of common bean pods. In contrast, the average ash content of common bean pods showed significantvariation among treatments ($p \leq 0,05$). Treatment P5 had the highest average ash content at 3,274 ± 0,425 grams, whilethe lowest ash content was observed in treatment P4. The combined treatment of ascorbic acid and compost tea led to increased ash content in common bean pods compared to treatments without this combination. Treatment P5 exhibited a 45,31% increase over P1, followed by treatment P2 with a 38,62% increase, P6 with a 22,68% increase, and P3 with a 16,2 % increase. Conversely, treatment P4 showed a 13,76% reduction compared to the control (Table 5).

Ash content reflects the total minerals present in plant material after the combustion of organic matter. It is inversely related to organic content, meaning that higher ash content indicates lower organic content. Common beans typicallyhave an ash content ranging from 0,36% to 0,5%, and the results obtained in this study do not align with this range [23]. Elevated ash content may be attributed to mineral-rich soil conditions, resulting in a higher accumulation of minerals in plant organs. Additionally, high ash content may signify the presence of undecomposed organic matter, such as crude fiber and lignin [24].

Table 5. Average wet weight, dry weight, organic content, and ash content of common bean pods per treatment

Treatment	Wet Weight (gram)	Dry Weight (gram)	Organic Content (gram)	Ash Content (gram)
P1	406,50 ± 38,066 ab	29,03 ± 3,192 a	26,777 ± 3,603 a	2,253 ± 0,183 ab
P2	483,80 ± 39,043 c	33,52 ± 3,269 a	30,393 ± 3,272 a	3,123 ± 0,503 cd
P3	422,60 ± 41,782 abc	29,83 ± 3,193 a	27,211 ± 3,393 a	2,618 ± 0,329 bc
P4	373,05 ± 37,880 a	26,41 ± 2,152 a	24,469 ± 2,687 a	1,943 ± 0,339 a
P5	443,90 ± 16,226 bc	31,18 ± 1,942 a	27,907 ± 2,276 a	3,274 ± 0,425 d
P6	390,70 ± 34,315 ab	28,16 ± 2,952 a	25,395 ± 2,996 a	2,764 ± 0,416 bcd

Notes: Numbers accompanied by the same letter in the same column show no significant difference in the 5% DMRT test.

3.3.2 Number of Pods, Pod Length, Pod Diameter, and Weight per Pod.

The production of bean pods varied significantly among treatments for the number of pods and pod length ($p \leq 0,05$), but no significant differences were observed in pod diameter and pod weight. Treatment P2 yielded the highest average number of pods at 84,25 ± 5,449 pods, while the lowest average was seen in treatment P4 at 70 ± 4,301 pods, which was not significantly different from treatments P6, P3, and P1. The combination of CT with AsA was found to increase pod production by 2,14% in treatment P6 and by 6,12% in treatment P5 compared to non-combination CT. Treatments P2 and P5 significantly outperformed P1 in terms of pod production. Average pod length revealed the highest response in treatment P1 at 22,97 ± 0,296 cm, while the lowest was in treatment P3 at 20,37 ± 1,248 cm. The data suggest that none of the treatments provided better results than the control. Average pod diameter and weight per pod also did not significant differences in responses (Table 6).

Common bean pod production is influenced by various factors, including pests, diseases, and climate. Disruptions during the flowering and pod formation process (V6, pod formation) can have a significant impact on the number of pods formed [25]. The application of AsA can enhance plant resistance to diseases and stimulate the production of auxin and gibberellin hormones, thereby promoting fertilization [22]. Pod length is primarily influenced by genetic factors inherited from the seed, with approximately 98.96% of pod length determined by the plant's genetics [26]. Pod diameter reflects the seed-filling process and serves as a quality indicator for a pod. It is predominantly influenced by both plant genetics and environmental conditions. Different common bean varieties can result in varying pod sizes [27]. Differences in pod weight can be attributed to variations in metabolic processes within plants, particularly in the translocation of assimilates and photosynthesis. Insufficient assimilate and photosynthate translocation to the fruit can lead to a decrease in fruit weight and number [26].

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Table 6. Average production of number of pods, pod length, pod diameter and wet weight per pod of common bean per treatment

Treatment	Number of Pods	Pod Length (cm)	Pod Diameter (mm)	Pod Weight (gram)
P1	74 ± 5,244 a	22,97 ± 0,296 b	6,549 ± 0,361 a	5,47 ± 0,516 a
P2	84,25 ± 5,449 b	21,39 ± 0,820 a	6,341 ± 0,465 a	5,75 ± 0,204 a
P3	73,5 ± 5,679 a	20,37 ± 1,248 a	6,295 ± 0,180 a	5,73 ± 0,458 a
P4	70 ± 4,301 a	21,26 ± 0,498 a	6,567 ± 0,304 a	5,34 ± 0,455 a
P5	78 ± 3 ab	20,785 ± 1,387 a	6,288 ± 0,083 a	5,70 ± 0,214 a
P6	71,5 ± 5,59 a	21,13 ± 1,005 a	6,318 ± 0,056 a	5,41 ± 0,438 a

Table 7. Productivity of common bean (*P. vulgaris*) per treatment

Treatment	Pod weight per plant (gram)	Productivity (ton/ha/cycle)
P1	50,81 ± 4,758 ab	6,83 ± 0,640 ab
P2	60,48 ± 4,880 c	8,13 ± 0,656 c
P3	52,83 ± 5,223 abc	7,10 ± 0,702 abc
P4	46,63 ± 4,735 a	6,27 ± 0,636 a
P5	55,49 ± 2,028 bc	7,46 ± 0,273 bc
P6	48,84 ± 4,289 ab	6,56 ± 0,576 ab

Notes: Numbers accompanied by the same letter in the same column show no significant difference in the 5% DMRT test.

3.4 Productivity

Data analysis of productivity indicated significant differences in results among treatments compared to control (P1), except for treatment P6. The estimated results revealed that treatment P2 achieved the highest productivity at 8,13 ± 0,656 tons/ha/cycle, while treatment P4 had the lowest productivity at 6,27 ± 0,636 tons/ha/cycle. Treatment P2 increased productivity by 19,03% compared to P1, followed by P5 with a 9,22% and P3 with a 3,95% increase. However, treatment P4 did not demonstrate the ability to enhance productivity compared to P1, with a lower productivity value of 3,95%. The combination of compost tea with AsA showed a 5,07% increase in productivity for treatment P5 and a 4,63% increase for treatment P6 compared to treatments without combination with AsA (Table 7). AsA has been demonstrated to significantly enhance the productivity of common beans during cultivation. Data analysis aligns with the findings of Gaafar et al., whose research established that treatment with 400 mg/L of AsA resulted in a remarkable 25.9% increase in vegetative growth and common bean seed production [4].

AsA functions as a highly efficient antioxidant, safeguarding crucial phytochemical processes within plants. The application of AsA through leaf spraying has a substantial impact on the levels of chlorophyll a, chlorophyll b, carotenoids, and total chlorophyll content. This increase is consistent with AsA ability to shield plant cells from the deleterious effects of Reactive Oxygen Species (ROS). The elevation in photosynthetic pigments such as chlorophyll a and b enhances the efficiency of photochemical reactions, ultimately leading to higher pod production [4]. The productivity values obtained from our data analysis range from 6.27 tons/ha to 8.13 tons/ha. These figures are by the potential productivity of Gypsy-1 common bean as described by the Ministry of Agriculture, which is 8 tons/ha. In another study concerning the application of KCl fertilizer at different doses, the average productivity of Gypsybean plants reached 8.94 tons/ha [28]. These results closely resemble our calculated productivity values, suggesting that treatments P2, P3, and P5 hold promise as viable alternatives to reduce the reliance on inorganic fertilizers.

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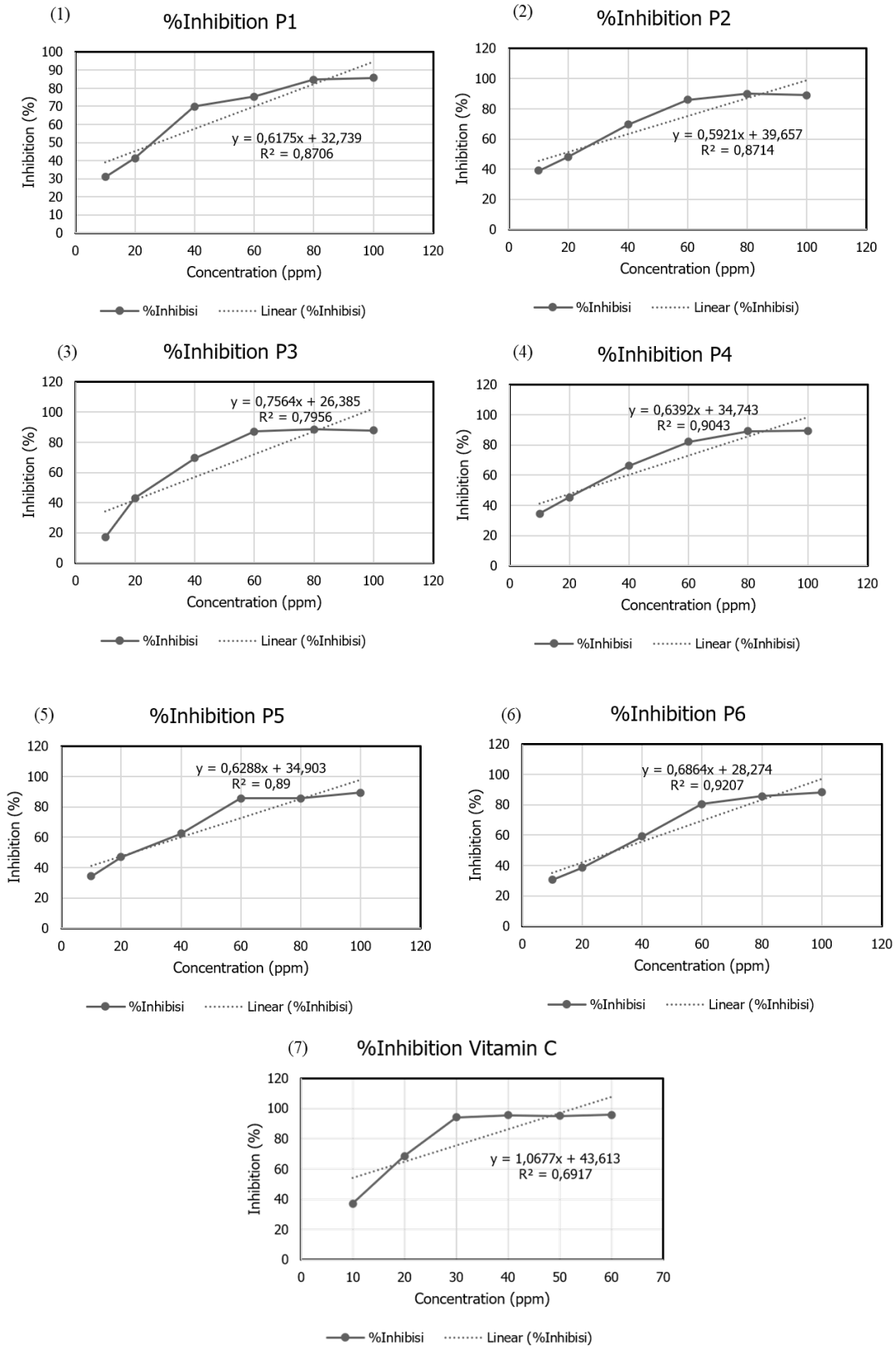

Figure 1. Free radical inhibition percentage curve of: (1) control treatment (P1), (2) ascorbic acid (AsA) treatment (P2), (3) manure compost tea (MCT) treatment (P3), (4) BSFR compost tea (BCT) treatment (P4), (5) MCT + AsA treatment (P5), (6) BCT + AsA treatment (P6), and (7) Vitamin C (positive control).

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Table 8. IC_{50} value for each tratment

No.	Test Sample Solution	IC_{50} value (µg/ml)	Classification of antioxidant activity
1.	P1	27,95	Very strong
2.	P2	17,46	Very strong
3.	P3	31,22	Very strong
4.	P4	23,87	Very strong
5.	P5	24	Very strong
6.	P6	31,65	Very strong
7.	Vitamin C	5,98	Very strong

3.5 Antioxidant Activity Assessment

Antioxidant activity is quantified through the inhibitory concentration 50 (IC_{50}), indicating the concentration of a substrate required to inhibit 50% of biochemical reactions. IC50 values are typically expressed in units of µg/ml, with lower IC50 values indicating higher antioxidant activity and greater suppression of free radicals [29]. The IC_{50} determination curves for each treatment group are depicted in the following figures (Figure 1-7).

The calculation of IC_{50} values for each treatment necessitates an analysis of the linear regression curve results. The highest IC_{50} value was recorded for treatment P2 at 17,46 µg/ml, while the lowest IC_{50} value was observed in treatment P6 at 31,65 µg/ml (Table 8). Remarkably, the combination of CT and AsA fertilizer exhibited a better 23.12% reduction in IC50 value for treatment P5. Conversely, treatment P6 experienced an increase in IC_{50} value by 32.59%. The combined application of MCT and AsA proved quite effective in enhancing the antioxidant activity of common beans, surpassing the results obtained without this combination. However, in the case of BCT, it exhibited better antioxidant activity when used alone compared to its combination with AsA. Interestingly, the antioxidant activity of common beans in both P3 and P6 treatments remained lower than that of P1, indicating that these treatments were not as effective in enhancing the antioxidant activity of common bean pods. This outcome may be attributed to the antibacterial properties of AsA, which might interfere with the stability of BCT, thereby reducing its effectiveness in enhancing common beans' antioxidant activity.

Antioxidant activity is categorized based on IC_{50} values as follows: very strong if IC_{50} < 50 ppm (1 ppm = 1 µg/ml); strong if IC_{50} is in the range of 50-100 ppm; moderate if IC_{50} is in the range of 100-150 ppm; weak if IC_{50} is in the range of 151-200 ppm; and very weak if IC_{50} > 200 ppm. A lower IC_{50} value indicates higher antioxidant activity [29]. The notable antioxidant activity of the AsA treatment can be attributed to the known antioxidant capabilities of vitaminC, acting as the positive control. Leaf spraying with AsA is known to stimulate the increased production of secondary metabolites in common beans, such as phenols, flavonoids, and tannins [4]. Elevated levels of these secondary metabolitescan significantly enhance the antioxidant capacity of commonbeans [3].

Vitamin C, or ascorbic acid, is a gluconic acid lactone compound derived from the combination of glucuronic acid and water-soluble ketolactone, featuring two ionizable hydroxyl groups. In nature, vitamin C exists in two isomeric forms: the reduced form (D-ascorbic acid) and the active chemically oxidized form (L-ascorbic acid), which can readily interchange. This property enables ascorbic acid to serve as a nutritional supplement with potent antioxidant effects [19]. The exogenous application of ascorbic acid to common bean significantly enhances the activity of both enzymatic andnon-enzymatic antioxidants (such as glutathione and ascorbicacid) in common bean. The increase in non-enzymatic and enzymatic antioxidants in common beans reinforces their defense against oxidative damage caused by ROS and elevates their tolerance to drought and salt stress [30].

4. Conclusion

This study reveals that the productivity response of common bean significantly differs for each treatment compared to the control, except for treatment P6. The highest productivity was achieved by treatment P2 at 8,13 ± 0,656 tons/ha/cycle, increased productivity by 19,03%, followed by P5 with a 9,22% increase, and P3 with a 3,95% increase compared to the control. However, treatment P4 did not demonstrate a significant increase and had a slight reduction in productivity by 3.95%. The combination of CT and AsA increased productivity by 5,07% for P5 and 4,63% for P6 compared to treatments without this combination.

The lowest IC_{50} value for the methanol extract of common bean pods was recorded for treatment P2 at 17,46 µg/ml. The combination of compost tea and ascorbic acid reduced the IC_{50} by 23,12% in treatment P5, while treatment P6

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increased the IC_{50} value by 32,59%. Treatment P5 effectively enhanced the antioxidant activity of common bean pods with the combination of AsA and CT, while treatment P6 exhibited better results without this combination. The IC_{50} values of treatments P3 and P6 were lower than those of the control, indicating insufficient enhancement of common bean pod antioxidant activity.

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Appendix

The supplementary data, including the raw data, can be accessed via the following link: https://drive.google.com/drive/folders/106tn5kHRMywPxT27RjP0eo0SDUnvtiuh

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The Influence of Polyethylene Glycol Precipitation Methods on Yield and Purity of White Radish Peroxidase

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Abstract

Proteins are widely used in various industries as highly valued biotechnology products. One example is horseradish peroxidase isolated from horseradish (*Armoracia rusticana*) that used as enzyme label in immunochemistry. However, the cultivation of horseradish is limited to subtropical countries, making the dependency on horseradish peroxidase unsustainable for tropical countries. Numerous studies have explored alternative peroxidases, and white radish peroxidase isolated from *Raphanus sativus* L. has emerged as a promising candidate. In this study, white radish peroxidase is isolated using the polyethylene glycol (PEG) precipitation method which is widely used as a simple and cost-effective method. This study aims to evaluate the effectiveness of the one-step and two-step PEG precipitation method. The one-step PEG precipitation method used in this study was done by mixing the white radish juice with PEG 6000 30% (w/v), while the two-step method was done by mixing it with PEG 400 20% (w/v) and PEG 6000 30% (w/v) consecutively. This study compares the yield and recovery levels of total protein and white radish peroxidase, as well as the enzymatic specific activity of white radish peroxidase isolated both by the one-step PEG precipitation and the two-step PEG precipitation. The results indicate that both extraction methods yield the same level of white radish peroxidase. However, they differ in terms of purity. The two-step extraction method results in white radish peroxidase with higher purity, as evidenced by its specific activity towards the chromogen ABTS in the presence of H_2O_2.

Keywords: *downstream processing, horseradish peroxidase, PEG, protein extraction, sequential extraction*

1. Introduction

Peroxidase is a significant biotechnology product with numerous diverse applications. The enzyme facilitates oxidative reactions or oxygen transfer between peroxides that serve as electron acceptors and the substrates that act as electron donors [1,2]. Horseradish peroxidase (HRP) is a widely used peroxidase in various sectors. It serves as an enzyme label in immunochemistry applications, such as in ELISA; as a catalyst for phenol removal in the bioremediation of wastewater; as bio bleaching and bio pulping in the paper industry, as well as decolorization of textile dyes [3–5]. Horseradish peroxidase is valued for its broad substrate specificity which allows it to oxidize an extensive range of chromogenic and chemiluminescent H_2 donors. It is also known for its ability to tolerate a wide range of pH and temperature, with optimum activity observed at pH 5.0-7.0 and 20-35°C [6,7]. However, the use of horseradish peroxidase may not always be a financially viable and sustainable option in certain parts of the world, such as Indonesia, because horseradish only grows in subtropical regions.

In recent years, researchers have been searching for a viable substitute for horseradish peroxidase. The alternative options include soybean peroxidase [8], red radish peroxidase [9], and white radish peroxidase [10,11]. White radish peroxidase is extracted from white radish (*Raphanus sativus* L.), which belongs to the Brassicaceae family--the same family as horseradish (*Armoracia rusticana*). It has been cultivated since 3000 BC and is now grown globally, including in tropical countries [12]. Research on peroxidase isolated from *Raphanus sativus* L. has been conducted since 1994, such as research on six isoperoxidases from Korean radish root [13], the isolation method & characterization of

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white radish peroxidase [10], immobilization of white radish peroxidase [14], and so on. Additionally, Barbosa et al. [14] found that white radish peroxidase shares about 70% of its amino acid chains with horseradish peroxidase. Given the global availability of *Raphanus sativus* L. and the structural similarity between white radish peroxidase and horseradish peroxidase, it could be used as a sustainable alternative to horseradish peroxidase.

There are several varieties of *Raphanus sativus* L. with different shapes and colors. In Indonesia, *Raphanus sativus* L. var. Hortensis Backer, also known as white radish, is cultivated as a horticultural commodity. White radish cultivation is distributed across various regions in Indonesia [15]. In 2020, the production of white radish reached 24,902 tons with an average yield of 15.96 tons/Ha. According to the Directorate of Food Corps [16], the average price for producers of white radish in 2022 was 492,975 IDR/quintal or approximately 5,000 IDR/kg. The use of white radish in Indonesia is currently limited to food consumption. Therefore, utilizing white radish for other purposes, such as in biotechnology, could increase its value.

Other researchers have developed several methods for extracting and purifying radish peroxidase that can be used as a reference, as can be seen on Table 1. Some of those techniques include the typical downstream processing which involves a multi-step cascade, beginning with protein extraction from the biological matrix, followed by clarification or extract separation from impurities, precipitation, and protein purification . Each extraction method has its advantages and disadvantages.

Ammonium sulfate precipitation is widely considered the gold standard method for protein extraction. However, it has some back draws, including low extraction rates, low yields, cumbersome operations, and purification difficulties [17–19]. On the other hand, PEG can be performed at ambient temperature, does not denature protein, and exhibits fast precipitation kinetics [20,21]. Many past studies utilized both features of ammonium sulfate and PEG in an extraction technique called aqueous two-phase system (ATPS) to extract peroxidase [22–24], and yet several back draws remain, such as high cost or optimization complexity [25]. While studies dedicated on improving extraction methods continues, looking back at conventional extraction methods might give positive points as its usually employ simpler steps and great result. For example, in 1968, PEG had been used to extracted and purified an enzyme through fractional precipitation to its crystalline state [26]. Therefore, polyethylene glycol (PEG) precipitation alone can be used as an alternative extraction method.

On past study, Apriliani [11] employed two-step PEG precipitation with overnight incubation time for each step of PEG 400 and PEG 6000 addition to white radish extract. This procedure is time-consuming, taking at least three days to obtain an impure white radish peroxidase extract. Therefore, this study presents a modified isolation procedure. The study compares the white radish peroxidase (WRP) extracted using two-step PEG precipitation using PEG 400 and PEG 6000 to the one-step extraction using only PEG 6000. The incubation time was reduced from overnight to 30-35 minutes for both methods. The reduction of incubation time was decided by considering its fast kinetic and inspired by the fractional precipitation done by Jansen et al. [26] that stirring the sample with PEG for 30 minutes only. Moreover, PEG incubation time does not significantly affect the yield [20]. This study aims to analyse the potential of modifying the established procedure for isolating white radish peroxidase to a simpler

2. Methodology

2.1 Experimental Design

The experimental design of this study is to compare two modified precipitation procedures using PEG as the precipitant. The experiment used white radish extract obtained from a single white radish which was bought from a local grocery. Each treatment is replicated six times. The incubation time was limited to 30-35 minutes. The concentrations of total protein and enzymatic activity of the white radish peroxidase (WRP) extract were analyzed with three to five replicates for each sample. As this study focuses on comparing one-step and two-step PEG precipitation effect on WRP yield, there is

Table 1. Radish Peroxidase Isolation Methods

RADISH PEROXIDASE SOURCE	EXTRACTION METHOD	PURIFICATION METHOD	REFERENCE
Cell suspension culture from white radish seed	Ammonium sulfate precipitation	Dialysis and DEAE-cellulose column chromatography	[10]
Red radish extract	Ultrafiltration using Amicon Cell with a membrane cut-off of PM10	Dialysis and DEAE-cellulose column chromatography	[9]
White radish extract	Two-step PEG precipitation using combination of PEG 400 20% & PEG 6000 30%	None	[11]

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no comparison between PEG precipitation method and other extraction methods.

2.2 White Radish Crude Extract Preparations

After washing the white radish (*Raphanus sativus* L.) with tap water, it was cut into small cubes. Next, phosphate buffer saline (PBS) was added, and the mixture was blended using a kitchen blender to obtain a smooth and homogenized white radish juice. The resulting mixture was then filtered through a cheesecloth, transferred into several Falcon tubes, and centrifuged at 10,000 rpm for 20 min at 4 °C, resulting in the separation of the white radish extract from cell debris. The supernatant was collected and used directly for the extraction of WRP . The resulting supernatant is referred to as white radish extract.

2.3 Preparation of samples & white radish peroxidase extraction

The white radish extract was divided into 12 Falcon tubes. Six of the tubes were used for one-step extraction samples, and the other six were used for two-step extraction samples. A small amount of white radish extract was taken from each tube for further analysis.

For the one-step extraction, approximately 30% (w/v) PEG 6000 was added to the final volume of each of the 6 tubes. The mixtures were then homogenized using a Falcon rotary machine for 30-35 minutes and centrifuged using the same procedure for white radish extract preparation. The pellet obtained from the centrifugation was then separated from the supernatant and gently rinsed with PBS to remove the remaining precipitant.

The six remaining tubes of white radish extract were used for a two-step extraction treatment. Approximately 20% (v/v) of PEG 400 was added to the final volume of white radish extract. The mixtures were homogenized and centrifuged using the same procedure as the one-step extraction. The resulting supernatants were transferred to new Falcon tubes, and a small volume of each tube was collected for further analysis. After that, approximately 30% (w/v) PEG 6000 was added to the final volume of each supernatant. The mixtures were homogenized and centrifuged again using the same procedure as before. The resulting pellets were then separated from the supernatant.

The pellets obtained from one-step and two-step extractions were resuspended with PBS consecutively for each treatment. A small amount of the pellet resuspensions from each treatment tube were collected for analysis.

2.4 Protein Quantification

The total protein of each treatment was measured using NanoDrop 280 nm. PBS was used as a blank. A standard curve was generated using BSA protein in the concentration range of 0.1678 - 1.678 mg/mL

2.5 White radish peroxidase enzymatic activity measurements

The WRP extract samples were diluted with PBS in the range of 50-100 times. Then, 10 µL of each diluted sample was added to a 96-well plate followed by the addition of 90 µL of ABTS solution containing 0.01% (v/v) H_2O_2. The ABTS solution used in this study is a 0.3645 mM (0.2 mg/mL) ABTS in sodium acetate buffer pH 4.5. The absorbances of the mixtures were measured every 5 minutes for 25 minutes using an ELISA plate reader spectrophotometer at a wavelength of 415 nm. The dilution factor was selected based on the linearity of the absorbance over time graph. The slope of the resulting graph was used to determine the unit peroxidase of the WRP extract.

One unit of white radish peroxidase is defined as the amount that increases the absorbance by 0.001 per minute of incubation at 415 nm. The concentration of white radish peroxidase concentration was calculated as follows:

$$Unit\ Peroxidase\ = \frac{Slope}{0.1}$$

$$WRP\ Concentration\ = \frac{Unit\ peroxidase}{Analite\ volume} \cdot\ Dilute\ factor$$

2.6 SDS-Page

The samples are then analyzed with SDS-PAGE using the Laemmli procedure [27]. The samples were loaded in 5% stacking gel and 10% resolving gel. The electrophoresis was run until the dye almost reached the edge of the gel.

2.7 WRP Lyophilization

The extracted WRP samples were lyophilized into a solid form. The resuspended samples were mixes with sucrose as lyoprotectant and PVP as wetting agent to minimize negative effects of lyophilization process. The lyophilization process was carried out for a day.

2.8 Specific activity comparison of WRP and HRP

An amount of lyophilized WRP was resolved in deionized water and the concentration was measured with NanoDrop using the same procedure as mentioned above. An amount of the commercially available HRP powder was resolved in deionized water in the same concentration as the resolved lyophilized WRP. The specific activities were measured using the procedure mentioned above (three replication data).

2.9 Statistical analysis

The protein quantification and enzymatic activity analysis data were statistically calculated. Normality, t-tests and two-way ANOVA were used to determine the data distribution and significance of each treatment. The parameters used for statistical analysis were protein recovery degree, white radish

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peroxidase recovery degree, and white radish peroxidase-specific activity.

3. Results and Discussion

3.1 Total protein recovery level

Total protein concentrations of white radish extracts and WRP extract were measured using Nanodrop. The protein recovery level of the final products compared to white radish extract is 10.33% and 5.17%, respectively (Figure 1). The two-step extraction shows a lower protein recovery level compared to the one-step extraction which means that fewer protein contaminants co-precipitated with the protein of interest.

One of the theories behind protein precipitation is the free volume exclusion effect [28]. It is influenced by various factors such as pH, temperature, molecular weight, and concentration of PEG used. Ignoring sample environments such as pH and temperature, protein precipitation using PEG mainly depends on PEG molecular weight and concentration as it directly affects the protein partition coefficient (Kp) needed for separating the protein of interest from other substances in solution. The protein partition coefficient (Kp) in PEG precipitation is defined as the ratio of protein concentration in the supernatant to that in the precipitate. A low Kp value indicates that the protein is present in the precipitate phase rather than in the supernatant phase. PEG with a molecular weight below 1540 is efficient in precipitating proteins with a low Kp. On the other hand, PEG with a molecular weight above 1540 can precipitate proteins with higher Kp values, as it reduces more water molecules through hydrophobic interactions between the PEG chain and the hydrophobic region of proteins [24]. Therefore, PEG with lower molecular weight can remove protein contaminants with a lower Kp value while retaining proteins with a higher Kp value in the supernatant.

The addition of PEG 400 20% (v/v) before to PEG 6000 30% (w/v) eliminates protein contaminants with a Kp value lower than white radish peroxidase by precipitating it. The remaining impurities were then eliminated by the addition of PEG 6000 by precipitation of the remaining higher Kp proteins, which in this case are white radish peroxidase and a few other protein impurities with similar K values.

3.2 White radish peroxidase recovery level

The white radish peroxidase was quantified in peroxidase units against the ABTS chromogen. The recovery level of white radish peroxidase from one-step extraction is 62.84%, which is slightly higher compared to two-step extraction at 56.65%. However, the difference is not statistically significant (Figure 2). Therefore, the extraction of white radish peroxidase by one-step extraction (PEG 6000 only) and two-step extraction yields the same amount of white radish peroxidase as the product.

This data shows that the addition of PEG 400 20% (v/v) to white radish extract does not necessarily precipitate or interfere with white radish peroxidase. The addition of PEG with a molecular weight higher than 1540 leads to peroxidase precipitation. According to the study conducted by Apriliani [11], white radish peroxidase started to precipitate after the addition of PEG 4000, and optimal precipitation was achieved

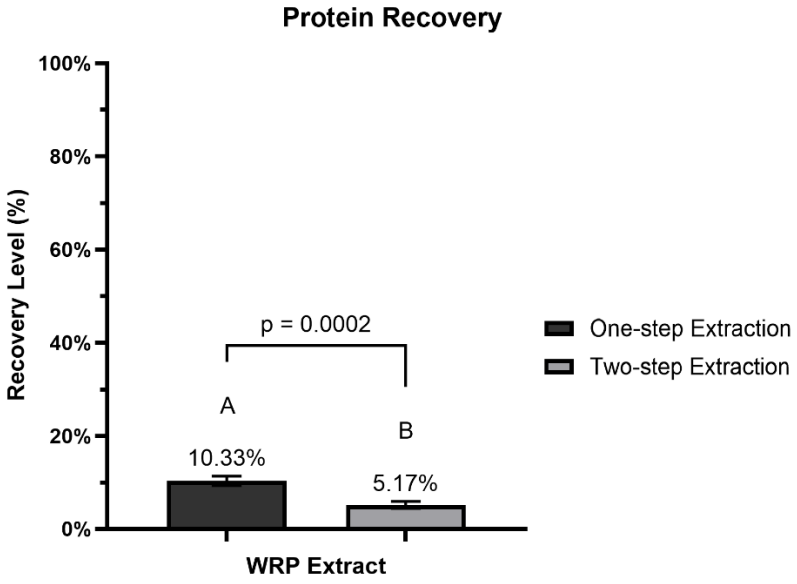

Figure 1. Protein recovery of one-step and two-step extraction compared to each method and its respective white radish extract. Statistical analysis using two-tailed t-test resulted p-value of 0.0002 between two experiments.

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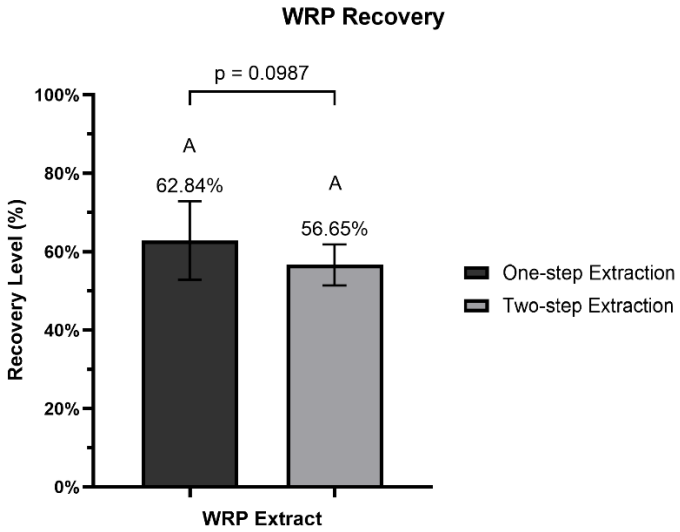

Figure 2. WRP recovery level in percentage compared to its initial white radish extract. Total white radish peroxidase was measured in unit peroxidase (UP), which indicates the amount of WRP capable of converting the ABTS chromogen and increasing the absorbance by 0.001 within one minute at a wavelength of 415 nm. Statistical analysis using two-tailed t-test resulted p-value of 0.0987 that indicates no differences in WRP yield between two experiments

with the addition of PEG 6000.

It is also known that PEG 6000 is commonly used to precipitate the protein of interest at different concentrations depending on its properties. Protein solubility decreases as the PEG concentration increases, resulting in protein precipitation [29,30]. As the equilibrium of protein solubility is affected, the Kp of white radish peroxidase is expected to decrease, leading to its precipitation. Although the Kp of white radish peroxidase was not measured in this study, according to Apriliani [11], 30% (w/v) PEG 6000 optimally precipitates white radish peroxidase. While 20% (w/v) PEG 6000 was able to precipitate about 41% white radish peroxidase, and PEG 6000 40% (w/v) precipitate around 66% white radish peroxidase with higher total protein as more protein contaminants co-precipitated.

3.3 White radish peroxidase specific activity & purity

The specific activity of white radish peroxidase was determined by measuring peroxidase units per milligram of total protein. The average specific activity of white radish peroxidase for one-step extraction is 523.61 UP/mg protein, while for two-step extraction, it is 941.01 UP/mg protein (Figure 3). Although there is no difference in the total amount of white radish peroxidase extracted by one-step or two-step extraction, there is a significant difference in the specific activity of white radish peroxidase as shown in Figure 3. The two-step extraction yielded white radish peroxidase with a specific activity 1.8 times higher than white radish peroxidase extracted with PEG 6000 alone. As the specific activity indicates the enzyme activity per total mg of protein in solution, it has a direct linear relationship with the purity of the white radish peroxidase. The use of PEG 6000 30% (w/v) alone (one-step extraction) did not result in a higher purity product because it has higher total protein, higher protein recovery, and lower specific activity compared to the two-step extraction using PEG 400 and PEG 6000 sequentially.

Another way to measure the purity of white radish peroxidase is to compare the specific activity of white radish peroxidase before and after the extraction process. As shown in Figure 4, the one-step extraction produced white radish peroxidase with a specific activity 6.16 times higher than the crude extract, whereas the two-step extraction was approximately 11.35 times higher. These results are consistent with the study done by Hammerschmidt et al. [21] where they optimized sequential precipitation of multiple mAb with a different pH range as the impurities were eliminated based on the pI value. In their study, the sequential precipitation which includes a two-step precipitation, helps to remove impurities in the first step and precipitate the final product in the second step. It also shows that sequential precipitation with two-step precipitation produces a yield with higher purity.

In most cases, the selection of the best extraction method depends on the extraction efficiency, expressed as the yield recovery levels [31]. However, in this case, since both methods gave the same recovery degree of white radish peroxidase, the efficiency of the extraction method was measured by comparing the specific activity of white radish peroxidase per mg of recovered protein. Figure 4 shows that the two-step extraction is almost two times more efficient than the one-step extraction in terms of white radish peroxidase purity and specific activity.

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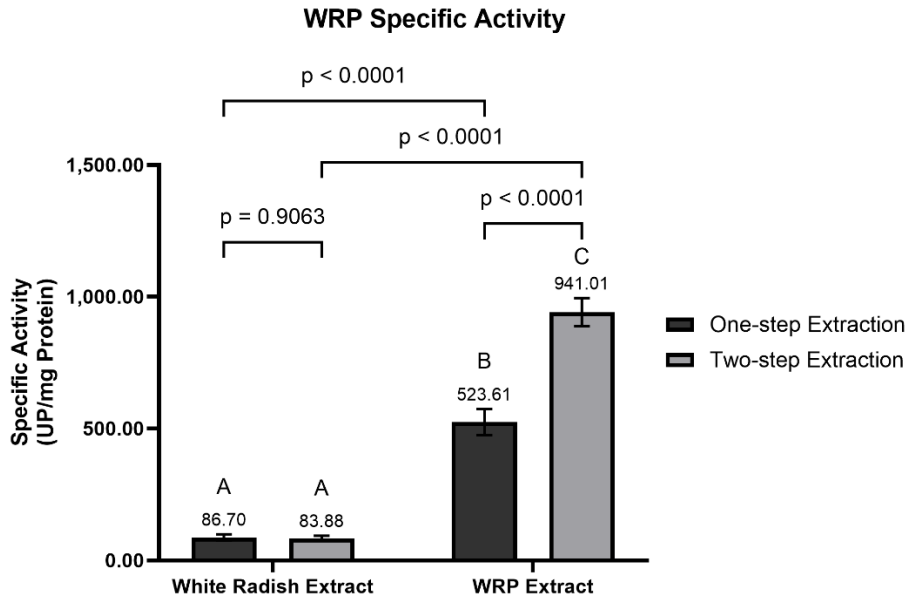

Figure 3. The specific activity of white radish peroxidase measured as unit peroxidase (UP) per mg of total protein. It was compared for each extraction method in both white radish extracts and WRP extract. Statistical analysis using two-way ANOVA resulted a p-value of 0.9063 for the initial specific activity of white radish extract (before enzyme extraction) that emphasized there is no difference between in starting material's specific activity value. While the comparison analysis of the specific activity of WRP extracts from one-step and two-step extraction resulted a p-value of <0.0001, indicates a significant difference between two conditions.

3.4 Molecular confirmation of white radish peroxidase isolate

The SDS-PAGE of WRP extract from both extraction methods shows the same amount of protein bands (Figure 5). Therefore, it indicates both samples contain the same proteins which support the white radish peroxidase recovery level regardless of the addition of PEG 400. The product solely depends on the addition of PEG 6000 which precipitates white radish peroxidase with several other protein contaminants of similar Kp values.

White radish peroxidase has several isoenzymes including the cationic and the anionic ones. Young Lee and Soo Kim [13] reported six isoenzymes with molecular weights of 31, 43, 43, 44, 45, and 50 kDa. Within all the bands that occurred on the gel, according to the migration and Rf values, the WRP extract might contain white radish peroxidase enzyme with a molecular weight indicated as 43 kDa. It can also be seen that there are many impurities in the sample. Based on band

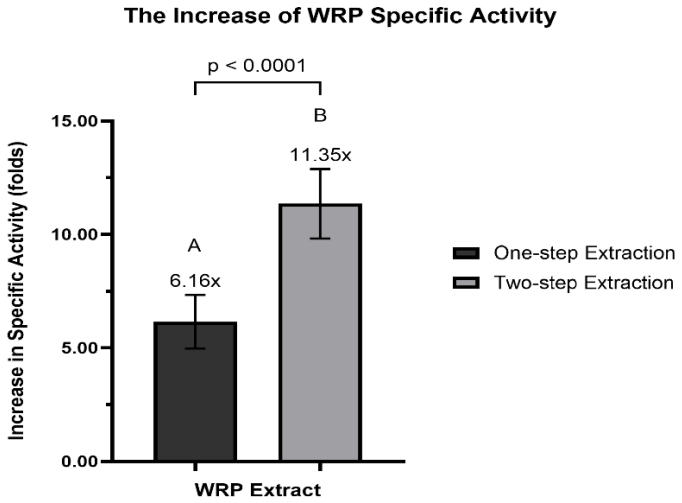

Figure 4. Comparison of the increase in specific activity of white radish peroxidase after extraction between one-step and two-step extraction with PEG precipitation. The increase in specific activity was measured by comparing the specific activity in WRP extract and white radish extract. Statistical analysis using two-tailed t-test results p-value of < 0.0001 that indicates significant differences between two experiments.

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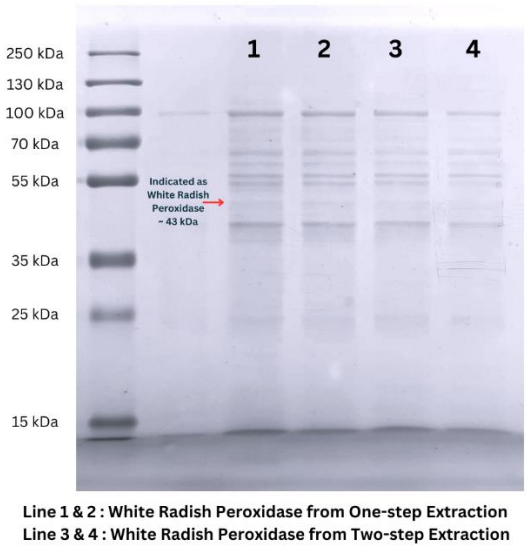

Figure 5. The SDS-PAGE protein band migrations of WRP from one-step and two-step extraction samples. Rows 1 & 2 represented WRP extract from one-step extraction samples, while rows 3 & 4 are from two-step extractions. Bands indicated as WRP were observed at 43 kDa.

intensity, the purity level of one-step extraction and two-step extraction respectively is 17% and 19%.

3.4 Comparison of WRP and HRP

In order to evaluate the ability of WRP as an alternative to HRP, the specific activity of powder form (lyophilized) WRP and HRP was compared. The results showed that the specific activity of lyophilized WRP cannot match the commercial powder form HRP with values of 2,093.59 UP/mg protein and 1,385,714.28 UP/mg protein respectively (Figure 6). The reason for WRP low specific activity is because of its low purity level whilst the commercial HRP is in high purity level. Moreover, the extreme condition of lyophilization might also affect the WRP enzymatic activity resulting in much lower value though it is not further analyzed in this study.

4. Conclusion

Simplification of the white radish peroxidase extraction method using only PEG precipitation with two-step extraction using 20% (v/v) PEG 400 and 30% (w/v) PEG 6000 consecutively demonstrates its potential as an alternative downstream process for white radish peroxidase extraction. The difference between one-step and two-step extraction also shows a significant difference in the yield of white radish peroxidase purity and specific activity. Further research is needed to include other parameters such as optimal pH, temperature, homogenization time, etc.

Conflict of Interest

The authors declare no competing interest in preparing this article. This research received no specific grant from any funding agency in the public, commercial, or non-profit sectors.

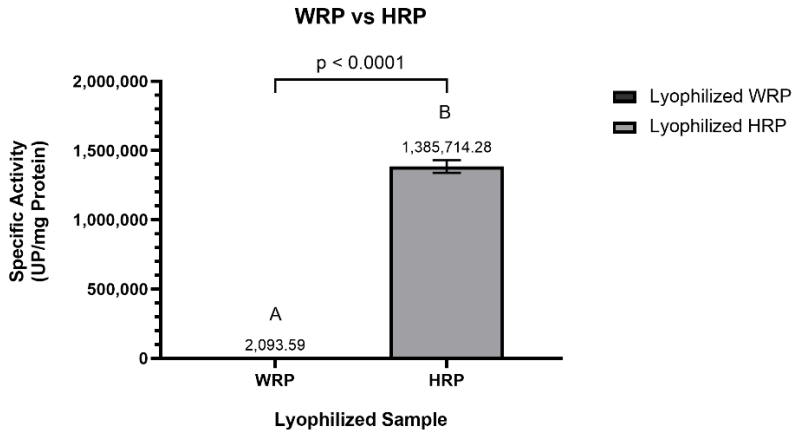

Figure 6. The comparison of specific activity of solid form WRP and commercial HRP. The extracted WRP was lyophilized first to turn it into solid form to match the available HRP form. Statistical analysis using two-tailed t-test results p-value of < 0.0001 indicated a significant difference between two comparisons.

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