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Full Length Article

Use of *Halimeda* sp. as Liquid Organic Fertilizer Enriched with *Trichoderma viride* Strain TV-15 Isolated from Soil

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Abstract

Halimeda sp. is a seaweed that is are underutilized despite being abundant in tropical areas such as Indonesia. Seaweed has the potential to be liquid organic fertilizer due to it containing essential nutrients, as well as plant growth substances, including auxins, cytokinins, gibberellins, which stimulate growth and increase crop production. On the other hand, *Trichoderma* sp. is a fungus that acts as a bio-activator, decomposer of organic matter, and controller of plant pests and diseases. The two factors combined are expected to complement each other as raw materials for liquid organic fertilizer, which improve soil structure and aid plant growth. This research was aimed at to determine the composition and content of seaweed-based organic fertilizers, some of which are enhanced with *Trichoderma* sp. isolated from soil. In this study *Halimeda* sp. was sampled from water, *Trichoderma* sp. was isolated from soil followed by morphology testing both macroscopically and microscopically and molecular testing of *Trichoderma* sp., and lastly, formulating liquid organic fertilizer (LOF) and its analysis. *Trichoderma* sp. was identified as *T. viride* strain TV-15. This species combined with *Halimeda* sp. LOF showed that both control and *Halimeda* sp. based LOF yielded a good level of organic carbon, iron and copper. These findings revealed that *Halimeda* sp. and *Trichoderma* sp. showed great potential as liquid organic fertilizers. © 2023 Friends Science Publishers

Keywords: Fertilizer; Growth; Seaweed; Soil; *Trichoderma* sp.; Plant growth substances

Introduction

As a maritime country, Indonesia has an extensive marine region with high potential and numerous biological resources such as seaweed. For example, *Halimeda* sp. is a seaweed with high economic value that has the potential to be developed further (Nazarudin *et al.* 2022). Furthermore, *Halimeda* sp. thrives in protected environments such as bays and grows in shallow water (Vogel *et al.* 2015; Ghosh *et al*. 2017). The thallus of *Halimeda* sp. resembles tiny sheets with a rough surface, rigid, greenish-white, and shaped like a branching kidney. Extracellular aragonite deposits were discovered in its thallus, with the majority of them containing $CaCO_3$, $MgCO_3$ and $SrCO_3$. However, *Halimeda* sp. is one of the underutilized seaweed species found in Indonesia. Seaweed has the potential to be used as organic fertilizer due to containing Fe, B, Ca, Cu, Cl, K, Mg and Mn, as well as plant growth substances such as auxins, cytokinins, gibberellins, which stimulate growth and improve crop yield (Zaman *et al.* 2015; Taberna *et al*. 2022). Study to utilize *Gracilaria* sp. and *Sargassum* sp. as liquid organic fertilizers (LOF) revealed that the liquid organic fertilizer derived from *Gracilaria* sp. and *Sargassum* sp. yielded 1.15% organic carbon (OC), 0.67 nitrogen, 0.45% phosphorus, and 0.48% potassium, with a pH level of 4.48 (Nasmia *et al.* 2021).

Organic fertilizers are classified as liquid or solid, depending on their form. Primary ingredients of organic fertilizer are derived from natural elements such as animal manure, animal body parts, and plants, which are high in minerals and have beneficial effects on soil fertility. LOF is a solution-based fertilizer that contains one or more soluble elements required for plant life (Phibunwatthanawong and Riddech 2019). LOFs are an alternative for plants to meet their macro- and micronutrient needs without producing pollutants (Ortiz 2020; Shaji *et al.* 2021). In addition, these fertilizers can deliver nutrients more quickly, allowing nutrient deficiencies to be corrected, thereby resulting in faster nutrient absorption (Ji *et al.* 2017). Thus, LOF is beneficial as it does not damage soil structure and can be used immediately without taking a long time to be absorbed by plants (Soeparjono 2016).

Trichoderma spp. are a group of rhizorferous fungi that live in the soil and act as organic matter decomposers, as well as plant pests and disease controllers (Tyśkiewicz *et al.* 2022). They are prevalent practically in all types of soils

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and ecosystems (Thapa *et al*. 2020). This fungus reproduces rapidly in plant roots. Many nutrients in the soil are soluble in small amounts or even insoluble, limiting nutrient cycling in the soil. Trichoderma has the ability to secrete organic acids that break down minerals and release nutrients into the rhizosphere (Abirami *et al*. 2022). Moreover, Trichoderma can degrade nitrogen molecules into simple nitrogen compounds such as NO_2. The presence of this fungus may allow the use of nitrogen-based fertilizers to be reduced.

Seaweed fertilizers are non-toxic and naturally decompose, especially making them environmentally benign (Prasedya *et al.* 2022). Several studies on seaweed fertilizer have been conducted (Soeparjono 2016; Ji *et al.* 2017). However, minimum levels of macro- and micronutrients from the liquid fertilizer have yet to be met because the levels are still relatively lower. Furthermore, the addition of EM4 as a bio-activator in seaweed fermentation is known to be unsatisfactory since various macro- and micro-elements in liquid fertilizer made from seaweed do not match the standards required by the Indonesian Ministry of Agriculture. Mold is known for being a significant producer of enzymes, which are expected to breakdown seaweed cell walls and release minerals for incorporation into liquid fertilizers (Baroud *et al.* 2021; Ammar *et al.* 2022). In addition, the bio-activator produced by *Trichoderma* sp. can accelerate the fermentation process and produce LOF (Raden *et al*. 2017).

Previous research has never utilized *Halimeda* sp. as a raw material for liquid organic fertilizer. The goal of this study was to create several liquid organic fertilizer formulations based on *Halimeda* sp. with *Trichoderma* sp. enrichment, with a control and three formulations. The preparation of LOF was achieved through experiments with various formulations, followed by testing based on numerous parameters to identify their contents. LOF from *Halimeda* sp. enriched with *Trichoderma* sp., being environment-friendly, is expected to promote plant growth and resistance.

Materials and Methods

Study area and species sampling

This study was carried out at Diponegoro University's Faculty of Agriculture and Animal Husbandry in Semarang, Indonesia. *Halimeda* sp., was collected in August 2022 from Jepara, Central Java, Indonesia (Fig. 1–2). Dry and aerated samples of *Halimeda* sp. were pulverized in a blender and sifted into flour. The fungal species *Trichoderma viride* strain TV-15 was isolated from the soil collected from tree root area at a depth of 10 cm. Soil sample (1 g) was weighed and transferred to a test tube with 10 mL of sterile distilled water and homogenized with a vortex for a few seconds. Next, the soil suspension was diluted up to 10^{-5} using the serial dilution method. A 0.1 mL of the last dilution was inoculated on Potato Dextrose Agar (PDA) medium. For this, 39 g PDA medium was dissolved in distilled water in a glass beaker filled up to the line of 1000 mL. Next, PDA medium solution was homogenized before heated to boiled. The mixture was then poured into six Erlenmeyer flasks of 250 mL and firmly closed using gauze. After that, the medium was sterilized in an autoclave for 15 min at 121ºC and 1 atm. The scatter plate method was used to inoculate the soil suspension with PDA that was put into a petri dish and solidified. The isolates were then cultured for 3×24 at 30ºC. During incubation, the observations were conducted every day at 1×24 h. Isolates exhibiting *Trichoderma* sp. features, such as light green to dark green colony color, hyphae reproduced quickly and evenly, and round-shaped colonies were purified and sub-cultured on a new PDA medium. Next, *Trichoderma* sp. fungus was purified and cultured on new PDA media, followed by incubation for 3×24 h at 30ºC. The pure colonies of *Trichoderma* sp. were obtained by isolating from other fungi (Fig. 3).

Trichoderma sp. identification

Morphological identification: Fungi were identified based on morphological characteristics and observed macroscopically and microscopically. Identification of *Trichoderma* sp. at the macroscopic level was done by determining colony color and growth rate. Furthermore, its characteristic was observed by looking at colony shape, margin, tip and color. Microscopic examination of was done by observing its hyphae, spores, sporangium, conidia and conidiophores under a microscope at 10×100 magnification. The hyphae were identified microscopically using Pitt and Hocking (2012) fungi identification key, as well as fungal identification guidebooks (Gadjar *et al*. 2000; Sastrahidayat and Rochjatun 2011).

Molecular identification: Molecular testing was done by transferring purified isolates (single colony) on slanted media in test tubes to the Genetics Science Lab in Tangerang, Banten, Indonesia. The molecular analysis began with fungal DNA isolation, amplification using an ITS1/4 universal primer, sequencing, and data interpretation (Martin and Rygiewicz 2005). Base sequences from sequencing findings were blasted or data matched with the NCBI database (Alshammari *et al.* 2021). Finally, the relationship of discovered fungal species was determined using NCBI data taking the highest similarity value, closest to 100% (Pearson 2013).

Formation of liquid organic fertilizer *Halimeda* sp. and *T. viride*

LOF by *Halimeda* sp. enriched with *T. viride* strain TV-15 were made using the following formulation:

H0 = *Halimeda* sp. + Aquades + Sugar + EM 4 (Control)
H1 = *Halimeda* sp. + Aquades + Sugar + EM 4 + H_2SO_4
H2 = *Halimeda* sp. + Aquades + Sugar + EM 4 + *T. viride* strain TV-15
H3 = *Halimeda* sp. + Aquades + Sugar + EM 4 + H_2SO_4 + *T. viride* strain TV-15.

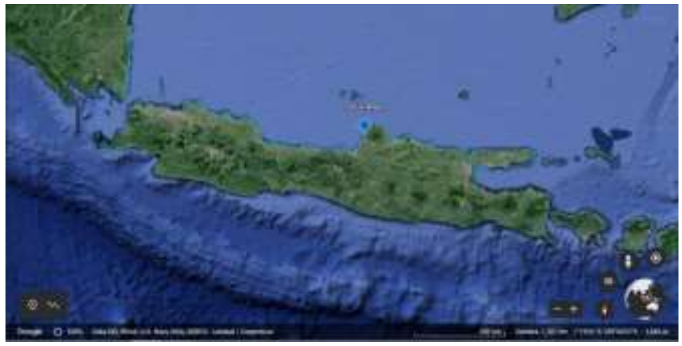

Fig. 1: Location of Awur Bay, Jepara, Indonesia; sampling location for seaweed Halimeda sp; (6º36'17"S, 110º40'16"E)

Fig. 2: *Halimeda* sp. seaweed samples

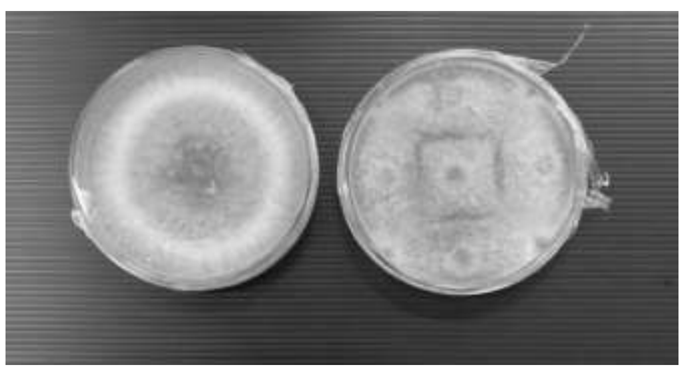

Fig. 3: *Trichoderma* sp. isolated from soil samples

Halimeda sp. flour was placed in a composter drum at a concentration of 250 g, and 0.2 M of H_2SO_4 1000 mL was added to hydrolyze the seaweed for 2 h before adding 25 mL of sugar, 500 mL of distilled water and 2.5 mL of EM4 to speed up the decomposition process. Next, the concoction was mixed until the mixture was equally distributed. The mixture was enriched with one cup of *T. viride* strain TV-15. Finally, the composter was completely sealed and left for 14 days (Tsaniya *et al*. 2021).

Analysis of liquid organic fertilizer using *Halimeda* sp. and *T. viride*

The LOF samples made by *Halimeda* sp. enriched with *T. viride* strain TV-15 were then tested at BBTPPI Laboratory (Central Center for Industrial Pollution Prevention Technology) Semarang, Central Java, to determine its concentration and composition. Several parameters of *Halimeda* sp. and *T. viride* strain TV-15 based LOF were tested, namely levels of OC (Walkey-Black method), pH levels (SNI 6989.11:2019), nitrogen (N) (SNI 2803: 2012 point 6.2), phosphorus (P) (SNI 2803.: 2012 point 6.3), potassium (K) as K_2O (SNI 2803: 2012 point 6.4.2), ferric (Fe) (AOAC 19th, 980.01, 2021, Ch.2, p.35), manganese (Mn) (AOAC 19th, 972.03, 2012, Ch.2, p.39) and copper (Cu) (AOAC 19th, 975.01, 2012, Ch.2, p.34). In addition, the populations of *Escherichia coli* (SNI ISO 7251: 2012), *Salmonella* sp. (SNI ISO 6579.: 2015) were also determined. The values of the above parameters in various treatments were compared with minimum technical requirements for LOF notified (No. 261/KPTS/SR.310/M/4/2019) by the Indonesian Ministry of Agriculture.

Results

Identification of *T. viride*

Macroscopic observations revealed that isolates recovered from soil samples had a dark green color, while hyphae were thick with a slightly yellowish dark green, light green, and white tint. The isolates exhibited *T. viride* features from this observation (Fig. 4). Observation showed that colony isolates had green hyphae, short phialides stalks, and round greenish conidia/spores developing at the ends. Conidia were growing in light green clusters. In addition, observations further revealed that the colonies had many conidiophore branches with longer branches underneath, and phialides were distinctly grouped, with 2-3 phialides in each group. This isolate had the morphological characteristics of *T. viride* (Fig. 4). The molecular test using ITS 1/4 primer identified the species as *T. viride* strain TV-15, with the identity of 97.57% (Fig. 5).

Analysis of LOF

Results of heavy metal testing on *Halimeda* sp. flour are shown in Tables 1–2. The amount of As in *Halimeda* sp. was relatively higher (4.970 mg/kg), closer to the Ministry of Agriculture's minimum technical standard for organic fertilizer (5.0 mg/kg). Hg levels was 0.152 mg/kg, which was lower than the highest quality limit for gum (0.2 mg/kg). Pb level was 0.020 mg/kg, much below the gum quality limit (5.0 mg/kg). Cd level as 0.005 mg/kg, which was also lower than the quality standard for gum (1 mg/kg). Cr was 0.074 mg/kg, but lower than the quality level for gum (40 mg/kg).

The analysis results showed that in treatments H0, H1, H2, and H3 the OC content was higher as compared to Commercial Seaweed Organic Fertilizer (CSOF) (Fig. 6). When compared with the minimum technical requirements for organic fertilizers, biological fertilizers or soil conditioners laid down by the Indonesian Ministry of Agriculture, LOF was still below the OC quality standard, namely at least 10%, so that the OC content in all treatments and CSOF was classified as very low and far from the quality standard of liquid organic fertilizer. The pH data in several treatments H0, H1, H2 and H3 obtained the same results (pH 7), while CSOF had pH of 8 (Fig. 6). Based on the minimum technical requirements of the Indonesian Ministry of Agriculture the pH values for all treatments for CSOF met quality standards i.e., 4–9. The N content in several treatments H0, H1, H2, and H3 showed lower results compared to CSOF, which was 0.78. Among the treatments, H0 and H1 obtained the highest results of 0.041 compared to the other treatments, namely H2 of 0.026, and H3 of 0.040. Based on the minimum technical requirements for LOF by the Indonesian Ministry of Agriculture, the quality standard for N content was 2-6%. Hence, all treatments and CSOF did not meet quality standards.

The results of the analysis of P content in treatments H0, H1, H2, and H3 showed lower results compared to CSOF, which was 0.04 (Fig. 6). However, in the H2 treatment the highest value was obtained, namely 0.008 compared to H0, H1, and H3. Based on the minimum technical requirements for LOF, laid down by the Indonesian Ministry of Agriculture, the quality standard for P content is 2–6%. So that all treatments and CSOF did not meet quality standards. The K (as K_2O) content in the treatments H0, H1, H2, and H3 showed lower results compared to CSOF, which was 0.41 (Fig. 6). However, among the treatments H0 obtained the highest value of 0.042 compared to H1, H2, and H3. Based on the minimum technical requirements for LOF laid down by the Indonesian Ministry of Agriculture, the quality standard for K content is 2–6%. So all treatments and CSOF did not meet quality standards.

The analysis of Fe content in treatments H0, H1, H2, and H3 showed higher results compared to CSOF. The treatment H3 obtained the highest value of 25.90 compared to H0, H1, and H2. Meanwhile, in CSOF, the Fe was not detected. The quality standard for Fe laid down by the Indonesian Ministry of Agriculture is 90-900 mg/kg. So all treatments and CSOF did not meet quality standards (Fig. 6). The Mn content in treatments H0, H1, H2 and H3 was higher as compared to CSOF. Among the treatments, H1 obtained the highest value of 22.4 compared to H0, H2, and H3, while in CSOF the Mn content was 0.40. Based on the minimum technical requirements for LOF, the quality standard for Mn content was 25-500 mg/kg. Hence, all treatments and CSOF did not meet quality standards (Fig. 6). The Cu content in treatments H0, H1, H2, and H3 were higher as compared to CSOF. In the treatment, H0 obtained the highest value of 0.497 as compared to H1, H2, and H3. In CSOF, the Cu content was 0.241. Based on the minimum technical requirements for LOF, the quality standard for Mn is 25-500 mg/kg. So that all treatments and CSOF did not meet quality standards for Mn (Fig. 6).

The *E. coli* population in treatments (H0, H1, H2 and H3) was higher; namely H0 (< 0.30), H1 (< 0.30), H2 (3.8), H3 (12) as compared to CSOF, in which *E. coli* was not detected. Based on the minimum technical requirements for LOF, the quality standard for *E. coli* was $<1 \times 10^2$ cfu/mL.

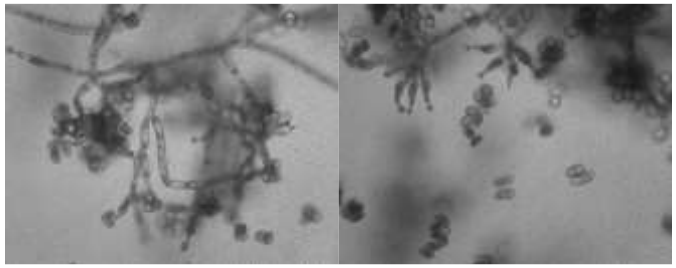

Fig. 4: Microscopical observations of *Trichoderma* sp. at a magnification of 10x100

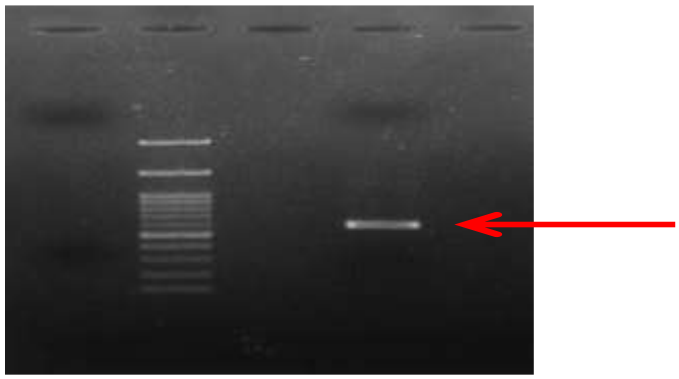

Fig. 5: PCR results for the *molecular* test of *Trichoderma* sp.
PCR Products (1 μL) Were assessed by electrophoresis with 0.8% TBE agarose; M, 1 00 bp DNA ladder (loaded 2.5 μL)

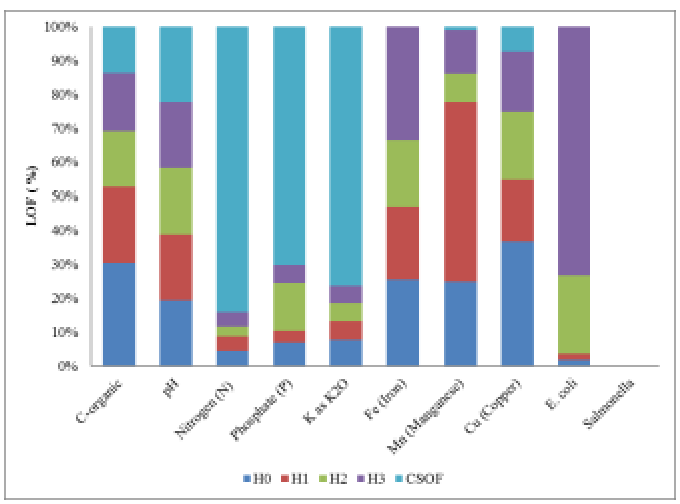

Fig. 6: Analysis result of liquid organic fertilizer *Halimeda* sp. and *T. viride* strain TV-15

Hence, all treatments and CSOF met quality standards (Fig. 6). The analysis of Salmonella population in several treatments H0, H1, H2, and H3 obtained negative results and CSOF was not detected (Fig. 6). Based on the minimum technical requirements for LOF, laid down by the Indonesian Ministry of Agriculture, the quality standard for Salmonella was <1 × 10^2 cfu/mL. that all treatments and CSOF met quality standards.

Out of several treatments, results showed that the measured parameters were not significantly different from each other. Based on a comparison of criteria with commercial organic fertilizers derived from other seaweeds, LOF derived from *Halimeda* sp. produced better results in terms of OC content, as well as Fe and Cu.

Discussion

Fertilizers made from natural materials, such as *Halimeda* sp., offered a significant possibility to mitigate the harmful impact of inorganic fertilizer overuse (Nasmia *et al.* 2021).

Table 1: Results of heavy metal testing on *Halimeda* sp. flour

Heavy wetal	*Halimeda* sp.	Quality standard
Arsenic (As)	4.970 mg/kg	Max. 5.0 mg/kg
Mercury (Hg)	0.152 mg/kg	Max. 0.2 mg/kg
Timbal (Pb)	< 0.020 mg/kg	Max. 5.0 mg/kg
Cadmium (Cd)	< 0.005 mg/kg	Max. 1.0 mg/kg
Chromium (Cr)	0.074 mg/kg	Max. 40 mg/kg
Standard deviation	2.456	2.561

Table 2: Analysis result of liquid organic fertilizer *Halimeda* sp. and *T. viride* strain TV-15

Parameters	Unit	H_0	H_1	H_2	H_3	CSOF
OC	-	0.550	0.404	0.296	0.311	0.25
pH	-	7	7	7	7	8
N	%	0.041	0.041	0.026	0.040	0,78
P	-	0.004	0.002	0.008	0.003	0,04
K	mg/kg	0.042	0.029	0.029	0.028	0,41
Fe	mg/kg	19.76	16.53	14.83	25.90	Not detect
Mn	mg/kg	10.58	22.4	3.501	5.524	0.40
Cu	mg/kg	0.497	0.246	0.270	0.241	0.10
E. coli	APM/100 mL	< 0.30	< 0.30	3.8	12	-
Salmonella	Colony/	Negative/25 mL	Negative/25 mL	Negative/25 mL	Negative/25 mL	-

Note : H0 (control), H1 (*Halimeda* sp. + H_2SO_4), H_2 (*Halimeda* sp. + *T. viride* strain TV-15), H3 (*Halimeda* sp. + H_2SO_4 + *T. viride* strain TV-15), OC (organic carbon), CSOF (Commercial Seaweed Organic Fertilizer)

Inorganic fertilizer use has resulted in groundwater pollution, soil degradation, and changes in soil microbial communities (Lin *et al*. 2019). One method that can be used as sustainable organic fertilizers is to use *Halimeda* sp. as a substitute for inorganic fertilizers (Muarif *et al.* 2022). This is because the components utilized to make this LOF are produced from organic materials, specifically *Halimeda* sp. In addition, *Halimeda* sp. contains carbohydrate molecule that affects the amount of organic matter in fertilizers. Organic matter is required to maintain soil fertility and pH through increasing aggregation and structure. In addition, organic matter can boost cation exchange capacity and water retention in the soil.

This study used the enrichment of the fungus *T. viride* has an important role in the decomposition process which can produce enzymes for biodegradation and become biocontrol agents. In this case the pH becomes an important factor affecting the activity of this fungus. An unsuitable pH can affect the synthesis of enzymes that are useful in the hardening process. The pH mismatch can prevent microorganisms from growing optimally and affect the calming results. In the research, fertilizer formulations were made to produce a pH that was in accordance with existing quality standards.

The OC content in liquid organic fertilizer produced was higher than commercial liquid fertilizer due to the addition of seaweed to the LOF produced. The addition of seaweed as a basic ingredient of OC adds nutrients containing amino acids so that the OC content in LOF will increase (Sundari *et al*. 2014). The results of helping the bacteria in LOF show that the content of bacteria such as *E. coli* and *Salmonella* is at a predetermined standard, so that the combustion process occuring in the fertilizer is not disturbed and later LOF is safe to use for plants because there are no bacterial pathogens that can cause harm to humans (Sundari *et al*. 2014).

Among other, N, P and K are indicators of limiting variables for tropical lowland forest plants growing on relatively fertile soils. N is abundant in the atmosphere and constantly circulates among plants, soil, water and air, requiring rapid plant growth and development. P is used for root development, seed formation, and disease resistance. and K promotes cell division in immature meristematic tissues of plants (Taiz *et al*. 2015). Soil pH is a critical feature that indicates the acidity and alkalinity of a soil solution, and it can forecast the availability of plant nutrients, toxins, and the activity of many key microorganisms (Pandey *et al.* 2020).

Mn is essential in Krebs cycle. It stimulates the activity of various enzymes, including oxidoreductase, hydrolase and lyase. Furthermore, isocitrate dehydrogenase, malate dehydrogenase, glycocyaminase, and D-alanyl synthetase are also produced from these processes. Mn is an essential component of water-splitting enzymes linked to photosystem II. Fe is a necessary and beneficial element (Taiz *et al*. 2015). In plants, Fe-containing protein is vital for cellular respiration, intermediate metabolism, oxygen transport, DNA stability and repair, and photosynthesis. Cu is a micronutrient required for growth and development. Though potentially toxic, it is also essential for electron transport chain respiration and photosynthesis, cell wall metabolism, ethylene synthesis, molybdenum cofactor biogenesis, and oxidative stress resistance. Furthermore, copper has antifungal qualities and has long been utilized in agriculture.

The LOF formulated here has various benefits for both plants and the environment. The benefits for plants are due to the OC content, and Fe and Cu are in accordance with the needs of plants for growth and development. For the environment, LOF are more environmentally friendly due to organic in nature and are not likely to change the order of ecosystems in the soil (Nasmia *et al*. 2021). Among the raw materials used for LOF, some are wastes, and can reduce the presence of waste so that the environment is cleaner, healthier and more balanced (Fernández-Delgado *et al*. 2022). This LOF uses raw materials that can be the best alternative in an effort to overcome the limitations of inorganic fertilizers. This is expected to reduce the use of chemical fertilizers, which may not be good for the environment and surrounding organisms. The LOF also contains *T. viride*, can maintain soil fertility due to its role as a decomposer, and thereby has a good impact on plants.

Conclusion

This study demonstrates that the use of *Halimeda* sp. as a primary ingredient with the enrichment of *T. viride* as a liquid organic fertilizer has promising agricultural applications. The OC content and the minerals Fe and Cu make liquid organic fertilizer based on *Halimeda* sp. advantageous compared to other commercial seaweed fertilizers. Based on several studies, seaweed contains minerals and contains growth regulators which are beneficial for plants and soil. This fertilizer when applied to plants, is expected to positively impact plant growth and yield. Use of *Halimeda* sp. can be as a base material for LOF and should be followed by additional research, due to its heavy metal content being below the highest limit of the standard set by the Decree of the Minister of Agriculture of the Republic of Indonesia No. 261/KPTS/SR.310/M/4/2019 on the minimum technical requirements for LOF.

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Author Contributions

AB Susanto and Wilis Ari Setyati proposed the research plan, processed the laboratory and field experiments and shared in writing the manuscript. Juwita Lesly Senduk, Dewi Basthika Makrima, and Dony Bayu Putra Pamungkas contributed field and laboratory assistants involved in this research.

Conflicts of Interest

The authors declare that they have no competing interests.

Data Availability

All new research results were presented in this article.

Ethics Approval

Not applicable

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