EXPLORATION OF BACTERIA Symbionts Mangrove Waste FOR THE PRODUCTION OF DECOMPOSTER

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ABSTARCT

Mangrove waste comprises of naturally decomposed dead mangrove leaves, twigs, and branches. This research aims to determine the types of bacterial symbionts in mangrove waste with potency as anti-bacterial agents. These anti-bacterial agents will subsequently be used in the production of compost with bio-activators. The research process involves isolation of symbiont bacteria, identification for symbiont bacteria with potency as anti-bacterial agent, DNA extraction using High Pure PCR Temperature Preparation Kit (Roche), DNA amplification by PCR 16s rDNA, and DNA Sequencing. Resulting amplified 16S rDNA are analyzed and then sequenced using Genetix program. Symbionts with identified anti-bacterial properties are used in bio-activator production. Samples of *Sargassum* seaweed are treated separately with resulting bio-activator product from the research and other bio-activator products for nutritional content comparison. Four types of symbiont bacteria are identified as potential anti-bacterial agents, namely *Pseudomonas sp., Flavobacterium sp., Acinetobacter sp., Bacillus subtilis*. It is further found that bio-activator products from mangrove waste have better quality compared to those found in the market and non-bio activator added liquid organic fertilizers. Therefore, bio-activators from mangrove waste is a potential alternative as natural bio-activator products.

Keywords: mangrove waste, symbiont bacteria, bio-activator, decomposer

Introduction

Mangrove is one of the many vegetation found in coastal areas of shallow bays, estuaries, delta, and protected beaches, and is highly affected by tides. After the Aceh tsunami disaster, restoration of mangrove grounds is intensely carried out in coastal areas all across Indonesia and is a program which receives special attention from the government of Indonesia. Among the essential functions of mangrove forests are: coastal barriers, prevention of sea water intrusion, natural habitat, feeding ground, nursery ground, spawning ground for many species found in

coastal areas. Moreover, mangrove forests also possess economic significance to the community living near coastal areas as a source of income, a source of industrial raw materials, and a source of various seeds.

The efforts of mangrove forest restoration often leave behind mangrove waste in the form of decomposed twig, leaves, and damaged seedlings. Mangrove waste is a significant issue since, in many instances, since there is no space allocation for containment. The existence of microorganisms associated with mangrove waste signifies activity of secondary metabolites synthesis similar to that of the host organisms. These microorganisms possess a significant potential in the exploration for new compounds. Mangrove waste symbionts bacteria can be defined as a colony of bacteria which grow, develop, and associate with mangrove waste. Microbes-associated bacteria also contribute in the nutritional cycle of the host and can be used as waste decomposers. Compounds produced by symbiont microbes have the potency as precursors in defense biosynthesis in metabolism against various pathogens and predators (Taylor *et al*., 2007). Isolated microbes from bio-active material producing plants have been known to possess high activity, even more so than their host plants (Krinsky, 2005). Based on the preceding findings, this research aims to discover symbiont bacteria of mangrove waste as anti-bacterial agents, and to utilize such properties as bio-activator in the production of compost with nutritional content.

Methods

Samples are collected from mangrove ecosystem area in the vicinity of Mangunhardjo, Tugu, Semarang. Mangrove waste samples are treated to isolate symbiont bacteria by means of dilution. The result is the analyzed for bacterial colony characteristics identification and bacteria purification by isolation of symbiont bacteria. Qualitative examination of symbiont bacteria against pathogenic bacteria *Vibrio alginolyticus, V. parahaenolyticus, V. vulnificus, V. harveyi, Micrococcus lutheus, Staphylococcus epidhemis, Pseudomonas aeruginosa, E. coli* is performed by overlay test method. Anti-bacterial test of mangrove waste symbiont bacteria against test bacteria is carried out using diffusion method, and morphological and biochemical identification of mangrove waste symbiont bacteria is performed. Amplification of DNA by PCR 16S rDNA follows, as seen in the research flowchart of Image. 1 below.

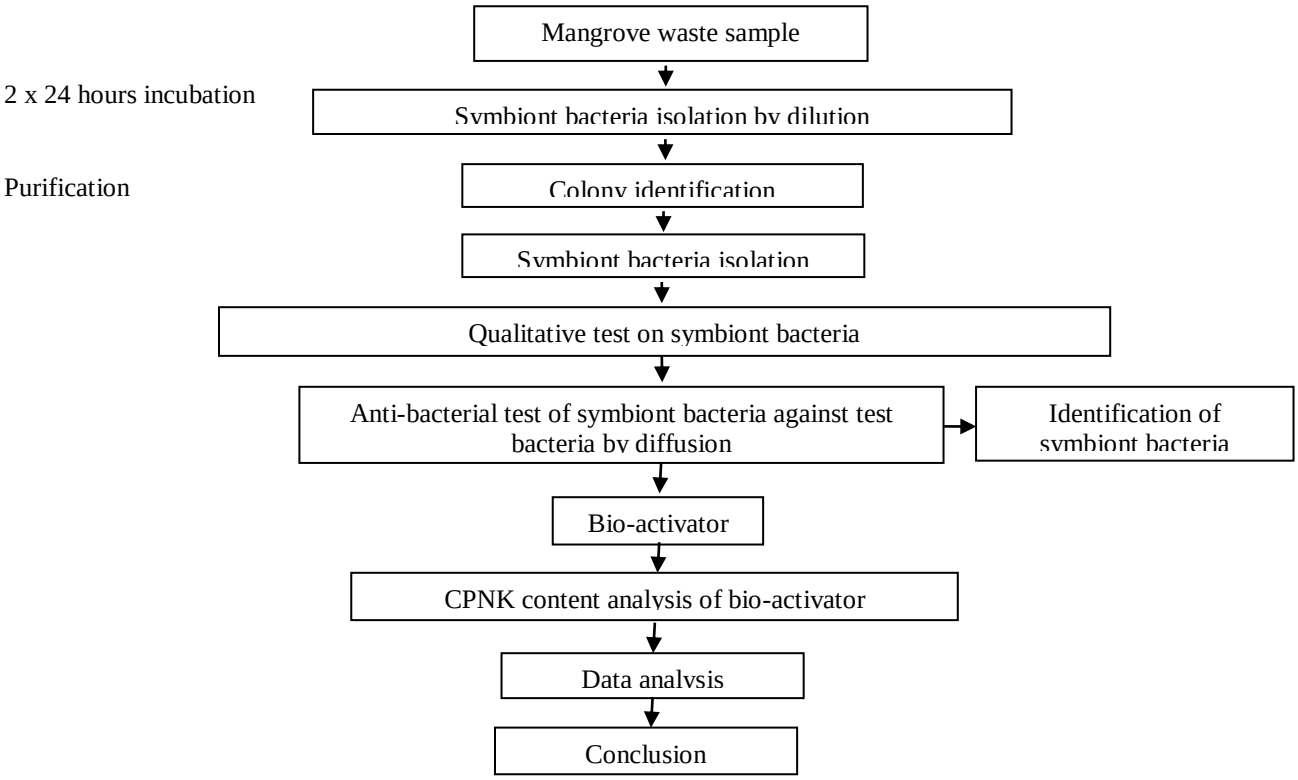

Image 1. research flowchart of mangrove waste symbiont bacteria

Morphological identification of bacteria includes observation of bacterial morphology and gram staining. Biochemical identification of bacteria involves oxidation test, catalyst test, pigment test, Indol test, motility test, H2S test, Hugh-Leifson (O/F) test, anaerobic test, and ADC test.

PCR 16s rDNA Amplification

DNA amplification is achieved using *Polymerase Chain Reaction* (PCR) 16s rDNA, 16S rDN, Electrophoresis and Visualization of PCR product, purification of PCR product, DNA sequencing, BLAST homology, and phylogenetic analysis. Bio-activator product is made of liquid media, namely Pepton (5g), beef extract (3g), and distilled water 1000 ml, which is mixed until homogeny state and then mixed into erlenmeyer 300 ml, sterilized in Autoclav for 15 minutes. The resulting mixture is inoculated and incubated in shaker with 150 rpm setting for 48 hours.

Growing bacteria in NB (Nutrient Broth) medium identified as the following: *Pseudomonas* sp., *Flavobacterium* sp., *Acinetobacter* sp., *Bacillus subtilis* are starters. Molasses, urea, water-added NPK are prepared to be harvested as bio-activator liquid production.

Analysis of CNPK content in Bio-activator

Analysis of CNPK content in bio-activator begins with the preparation of *Sargassum* sp seaweed as test samples for comparative study of bio-activator product of this research and other products (EM4). The samples are fermented for 14 days. pH measurements are carried out regularly during the process of fermentation. After 14 days, samples are tested for nutrient contents of C-organic, nitrogen, phosphor, and potassium. Statistical data of fermented samples are performed by normality, homogeny, and ANOVA test for F value prior to concluding the results of the tests. Flowchart of the CPNK content analysis of bio-activator is given in Image 2 below.

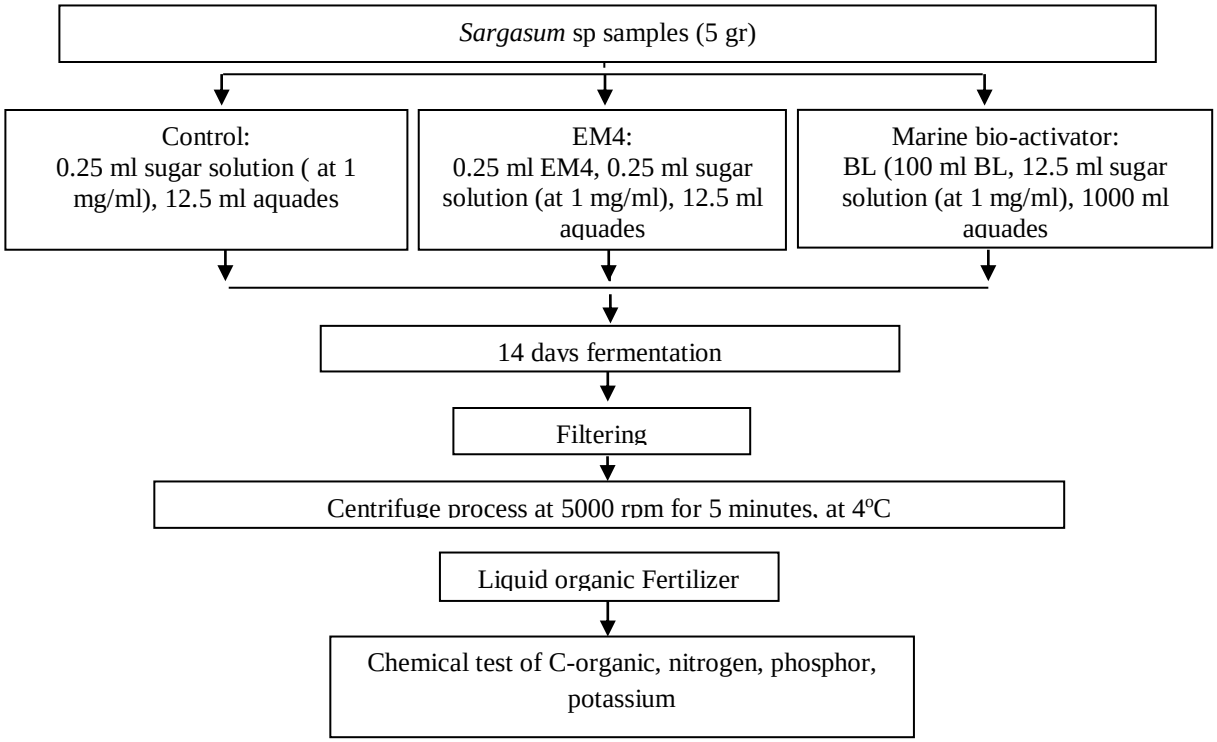

Image 2. Flowchart of the CPNK content analysis of bio-activator

C-Organic Test

C-Organic test in Black (1965) utilizes carbon as organic compound to reduce orange-colored Cr_6^+into green-colored Cr_3^+ in an acidic environment. The intensity of the resulting color green is proportional to the existing carbon content and is measurable by the utilization of a spectrophotometer with 561 nm wavelength.

Nitrogen Test

The test is carried out by using nitrogen, as an organic compound, which is oxidized in a thick H_2SO_4 environment with selenium solution as catalyst to form $(NH_4)2SO_4$. During the distillation process, the extract is turned into base by adding NaOH solution. Freed NH_3 is then bound in boric acid and mixed with standard H_2SO_4 solution. Nitrogen content is measured according to Hjeldahl method using spectrophotometry (Page, 1982).

Phosphor and Potassium Test

In phosphor test, reserve phosphor is fixated with HCl 25% reagent. The reagent will dilute phosphate and potassium compound forms with approximate of total-P and total K amount. Phosphate ion will react with ammonium molybdate in acid environment to form phosphomolybdate acid. The acid is then put into reaction with ascorbic acid, resulting in blue molybdate solution. Color intensity of the resulting blue solution is measured using a spectrophotometer at 693 nm wavelength, whereas potassium content is determined using a flamephotometer (Sudjadi *et. al*., 1971).

Results and discussions

Isolation of Mangrove Waste Symbiont Bacteria

16 isolates of bacteria are made in determining symbiont bacteria of mangrove waste in this research. The characteristic of each colony of the isolate bacteria is determined based on its color, shape, texture, and edges. Isolates of symbiont bacteria found in *Rhizophora mucronata* are mostly white in color. The most prevalent shape found is circular, and the most dominating texture of the isolates observed is convex. From 16 isolates identified, four are chosen as the

focus of this research. The choice is determined based on the size of inhibition zone resulting from the anti-bacterial activity of the isolate bacteria.

Identification of symbiont bacteria of mangrove waste

Identification the symbiont bacteria in chosen isolate samples is carried out using biochemical test as described in Cowan and Steels (1974) and Bergey (2005). One isolate is put through DNA amplification using PCR 16s rDNA. Results of biochemical test are presented in the following Table 1.

Table 1. Results of biochemical test on three isolates

Biochemical test	Isolate 1	Isolate 2	Isolate 3
Gram	-	-	-
Shape	Long-rod	Short-rod	Short-rod
Oxidation	+	+	+
Catalase	+	+	+
Pigment	+	+	-
Indole	-	-	-
Motile	+	-	-
H_2S	-	-	-
OF	-	-	-
Anaerob	+	-	+
ADC	X	X	-
Name of bacteria	***Pseudomonas* sp**	***Flavobacterium* sp**	***Acinetobacter s*p**

Three species of bacteria, namely *Pseudomonas* sp, *Flavobacterium*sp and *Acinetobacter* sp, are identified in the biochemical test of the three symbiont bacteria isolates taken from mangrove waste.

Identification by DNA amplification using PCR 16s rDNA

DNA amplification by PCR 16S rDNA shows positive result with the formation of DNA profile of the isolate bacteria with >1000 bp length. Primer used in the procedure is universal primer 27F and bacteria-specific primer 1492R for eubacteria. Sequencing process performed results in base sequence of the isolate 4 (>1000 bp), as displayed in Image 4.

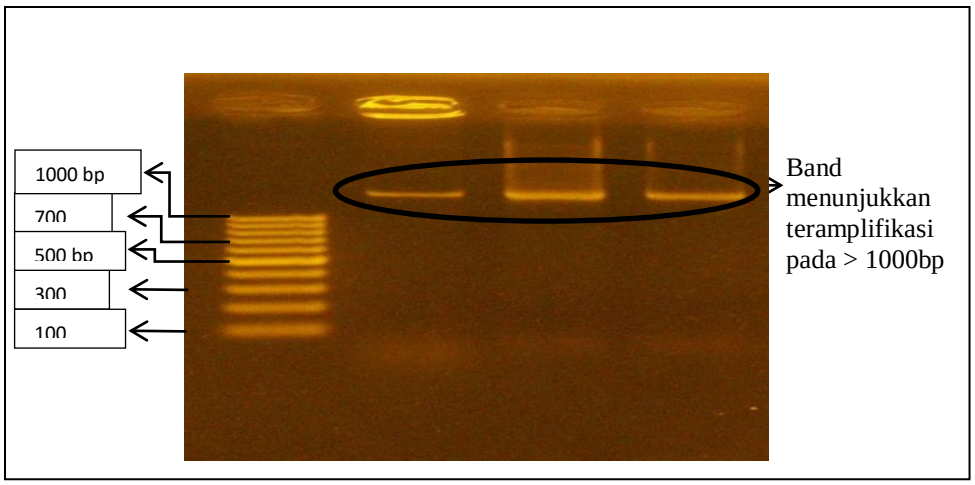

Image 3. Visual of Result Band of PCR 16S rDNA on Isolate Sample 10.B.2

CCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTC
GGCTACCACTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGA
GGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCT
GACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCG
GTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAA
TTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGG
GAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCG
ACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT
GAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGA
AACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGA
CATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGA
TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAA
CCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCAG
CGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTA
GTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAA
GTCGGTGAGGTAACCTTTTAGGAGCCA

Image 4. Base Sequence Result of PCR 16S rDNA with Primer 27F on Isolate 4

Resulting DNA sequence is identified to the DNA sequence stored in DNA database bank. Searching process is carried out via internet using *Basic Local Alignment Search Total* (BLAST) program in *National Center for Biotechnology Information, National for Health,* USA (www.ncbi.nlm.nih.gov). Analysis result of phylogenetic tree of isolate 4 is presented in Image 4. Homology identification result of isolate 10.B.2 with BLAST searching is displayed in Table 1.

BLAST homology identification of isolate 10.B.2 bacteria shows 99% match with the bacteria *Bacillus tsubtilis.*

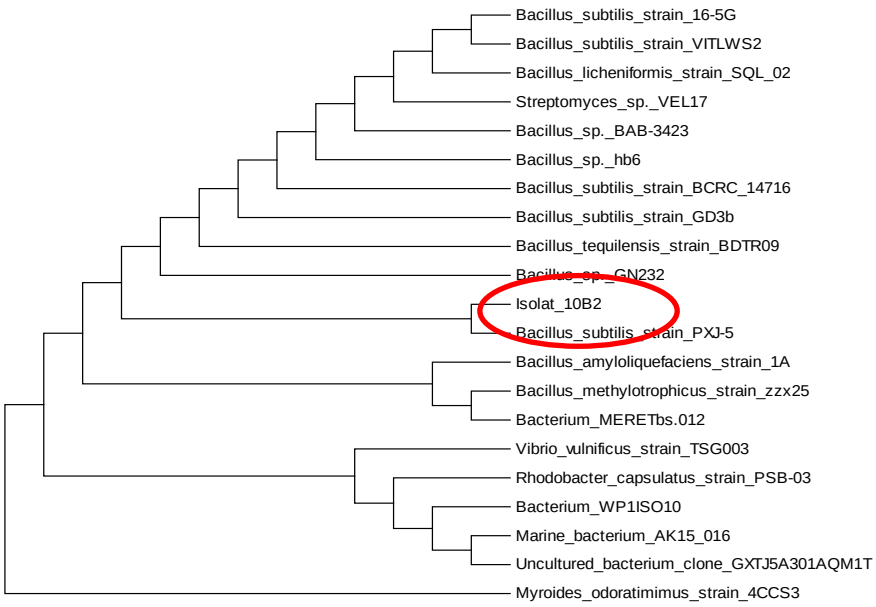

Image 5. Phylogenetic Tree and Sequencing Result of 16S rDNA of Isolate 10.B.2

Qualitative Test of Mangrove Waste Symbiont Bacteria and Test Bacteria

Qualitative test of symbiont bacteria of mangrove waste against test bacteria *Staphylococcus epidhemis* (MDR), *E. coli* (MDR), *Vibrio alginolyticus*, *V. parahaenolyticus*, *V. vulnificus*, *V. harveyi*, *Micrococcus lutheus*, *Enterobacter* (MDR), *V. Harvey*, *Pseudomonas aeruginosa* finds that *Pseudomonas* sp shows the highest activity (10,73 mm) against *Vibrio vulnificus*.

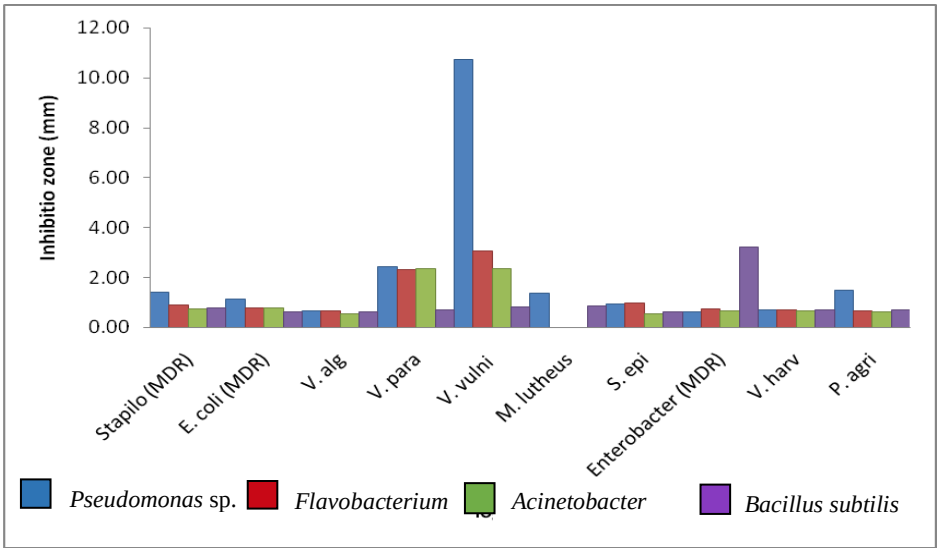

Image 6. Result of qualitative test on mangrove waste symbiont bacteria against pathogenic bacteria

B. subtilis bacteria is shown to be at peak activity (3,06 mm) against *Enterobacter* (MDR), whereas *Flavobacterium* shows highest activity against *V. vulnificus*. Inhibition zones produced from activities of other symbiont bacteria against pathogenic bacteria are measured at less than 2.5 mm.

C-Organic, Nitrogen, Phosphor, Potassium contents

Data from the measurements of C-organic and nitrogen (%) as well as phosphor and potassium (ppm) content in liquid fertilizer made of *Sargassum* sp are presented in Image 7 and Table 2. Based on test results, the highest percentage of C-organic content found at control is measured at 3.83%±0.05. The highest value for nitrogen content is found in solution with 1.44%%±0.04 addition of marine bio-activator. Result of phosphor content test in marine bio-activator found the highest percentage of content in the addition of 170.86ppm±0.66. The highest potassium content is found in the 938.83±0.51 addition of EM4.

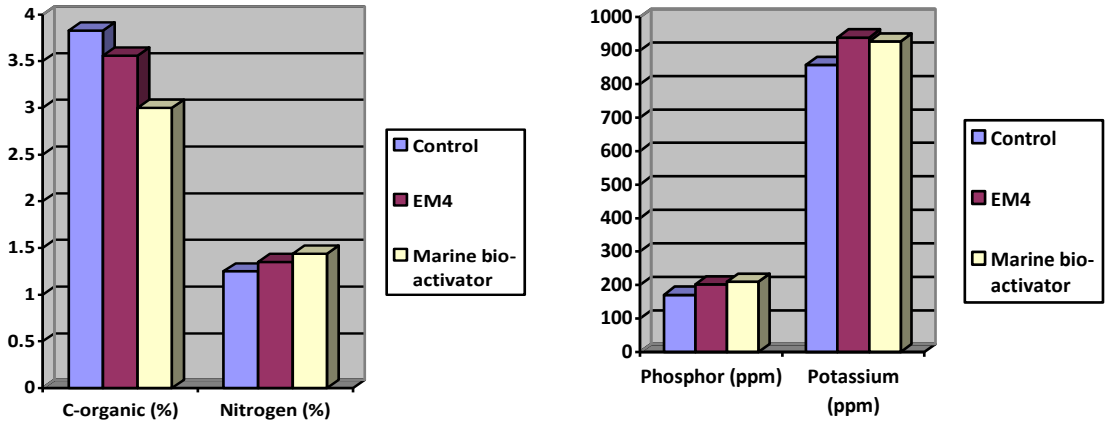

Image 7 Test result of C-organic, nitrogen (%), Phospor and Potassium (ppm) content.

	C-organic (%)	STD (±)	Nitrogen (%)	STD (±)	Phosphor (ppm)	STD (±)	Potassium (ppm)	STD (±)
Control	3.83	0.05	1.25	0.04	170.86	0.66	856.98	0.67
EM4	3.56	0.06	1.35	0.03	201.98	0.79	938.83	0.51
Marine bio-activator	3.00	0.04	1.44	0.04	209.82	0.55	926.76	0.41

Table 2. Test result of C-organic, nitrogen (%), Phospor and Potassium (ppm) content.

BNJ test result shows significant difference in the content of C-organic, nitrogen, phosphor, and potassium of *Sargassum* sp liquid fertilizer with control treatment, EM4 treatment, and 95% marine bio-activator treatment.

Discussion

As seen in table (?), EM4 treatment of liquid fertilizer results in 3.56% of C-organic content. This content value is 0.27% lower when compared to that of control treatment, with 3.83% C-organic content. Treatment using marine bio-activator yields a C-organic content value of 3.00%, or 0.83% lower than the amount found in control treatment and is 0.56% decrease compared to that of the EM4 treatment. This confirms the activity of decomposition of C and organic materials during the compost making process. Hakim (1986) added that organic materials are broken down into various nutrition for plants, such as N, P, K, Ca, Mg, S, Fe, Mn, B, Cu, Zn, Cl and other cell building compounds. These organic compounds are essential for the survival of microbes.

C-organic content obtained from marine bio-activator treatment is shown to be lower since the microbes involved in the decomposition process perform more effectively. Blum *et al* (1988) wrote that bacterial activities can increase the availability of nutrition through nitrogen mineralization and assimilation process. Moreover, Martinko and Madigan (2005) proved that bacteria thrive and develop in dead organism by decomposing organic compounds such as protein, carbohydrate and fat through single metabolism process like amino acid, methane, CO2, C, N, H, O_2, P, S gases or inorganic compounds such as K, MG, Ca, Fe, Co, Zn, Cu, Mn dan Ni.

Nitrogen content increases 0.25% in EM4 and 0.19% in marine bio-activator treatments. This confirms the findings that organic materials decomposition increases nitrogen content. Indriani (2005) supports this finding by confirming that decomposition process lowers or deplete the carbohydrate content and increases the N (amonia) content in the dilution.

The increase in nitrogen content of each treatment is attributed to the activities of microorganisms during decomposition process which break down organic materials. Sundari *et.al*. (2012) wrote that effective microorganism (EM4) reinforces organic materials decomposition.

Nitrogen content found in the marine bio-activator treatment is shown to have better value than that in control and EM4 treatment. This phenomenon is caused by more optimal and specific performance of bacteria in increasing the nitrogen content during the decomposition process. Marine bio-activator contains fewer decomposer bacteria species than the number of EM4 bio-activator. Bacillus, Pseudomonas, Acinetobacter dan flavobacterium are found in the marine bio-activator. These bacteria have been confirmed to have specific nature in increasing the nitrogen as an organic nutrient. Nasahi (2010) agrees to this finding in stating that facultative anaerobic bacteria such as Bacillus are non-symbiotic, nitrogen-fixating bacteria. In addition, nitrogen fixating bacteria survives on the availability of nitrogen, inorganic nutrition, energy source, high humidity level and appropriate temperature.

Phosphor test yields notable results in this research. Optimal fermentation time contributes greatly to the accuracy of the results. Fermentation or composting time plays a vital role in addition to the concentration of bio-activator used in the formation of nutritional content of final product.

The choice of ingredient in the production of a liquid fertilizer is closely related to the phosphor content of the product. The main ingredient in this research is *Sargassum* sp, which is a family of brown seaweed with rich phosphor content. Kalaivanan *et al.* (2012) states that brown seaweeds possess abundant organic material.

The usage of bio-activators in production process also determines the nutritional content of liquid fertilizer products. Comparison of nutritional content between fertilizers with marine bio-activator and EM4 bio-activator additives shows no significant difference. The difference in contents in the two bio-activators is attributed to this result. Phosphor content found in EM4 and marine bio-activators are found at 136,78 ppm and 90,48 ppm respectively.

Phosphor content of the final product is measured at different levels between that with marine bio-activators and that with EM4. The final product resulting from marine bio-activator use is proven to yield higher phosphor content than that from EM4 use. The role of symbiont bacteria in marine bio-activator, with their nitrogen-fixating specific activities, results in high phosphor fertilizer. The most contributing species is found to be Pseudomonas, which is known to be a species with high nitrogen-fixating activity. This finding is in line with Nasahi (2010)

who states that phosphate diluting microbes includes fungi and bacteria such as Pseudomonas and Bacillus.

The usage of EM4 bio-activator in the production process of fertilizers results in product with better potassium content than the use of marine bio-activator. The fact that EM4 bio-activator contains richer potassium content than its marine counterpart highly contributes to this fact. Potassium contents found in EM4 and marine bio-activators are 8,403.70 ppm and 537,25 ppm respectively. Therefore, EM4 bio-activator works more optimum in producing fertilizer products with high potassium content than the marine bio-activator.

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