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A large graphic at the top of the page shows a molecular structure. It features a green and red ball-and-stick model of a complex organic molecule, possibly a protein or a large organic compound, set against a blue background with a grid of small dots. The molecule is partially enclosed by a red wireframe mesh. The overall shape of the graphic is a large, curved, triangular area that tapers to the left.

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Analisis of Phytoplankton Diversity on the Productivity of Vannamei Shrimp (*Litopenaeus vannamei*) Intensive Pond, Jatisari Village, Banyuwangi

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Abstract

Phytoplankton can be used to estimate the potential for vannamei shrimp production. It can be used as a provider of nutrient sources and has an important role in improving water quality. This study aims to analyze the phytoplankton community structure on the productivity of intensive vannamei shrimp ponds. The research was conducted in Jatisari Village, Banyuwangi, East Java, Indonesia. Carried out parameters were the calculation of density, phytoplankton diversity index, culture performance (SR, FCR, ADG), and water quality (temperature, water transparency, salinity, pH, DO, NO₂, NO₃, PO₄, NH₄). The results showed that there were six classes and 33 genera from both ponds, Chlorophyceae (10 genera), Bacillariophyceae (8 genera), Cyanophyceae (9 genera), Dinophyceae (3 genera), Euglenophyceae (1 genus), Cryptophyceae (1 genus). The index value of the two ponds shows moderate diversity, H' pond 1 is 1.76, and pond 2 is 2.02. The two plots' cultivation performance was SR 92% and 80%, FCR 1.08 and 1.13, ADG 0.31 g.day⁻¹ and 0.35 g.day⁻¹, respectively. The physical and chemical parameters of the research showed a good enough value for the life of vannamei shrimp and phytoplankton.

Keywords: Cultivation performance, Phytoplankton, Vannamei shrimp.

INTRODUCTION

Indonesia is the third-largest seafood consumer country after China and Japan, Indonesia is the fourth largest shrimp exporter after India, Ecuador, and Argentina [1]. Vannamei shrimp is an aquaculture species whose production has increased the fastest over the past five years [2]. Indonesia can produce shrimp up to 919,959 tons [3]. Production of vannamei shrimp in East Java in 2017 was 156,139 tonnes. This value is higher than other provinces in Indonesia [3].

To increase production yields, many cultivators adopt an intensive cultivation system, characterized by probiotics, commercial feed with high protein content, and stocking densities ranging from 100-300 individuals.m⁻² [4]. The higher the stocking density, the more metabolic waste will increase [5]. The distribution of feed that is used and not utilized by shrimp was controlled [4] so that shrimp feed in intensive cultivation systems becomes the main source of producing organic material, which can cause nutrient overload. Organic material in an amount suitable for carrying capacity has a positive impact because it can benefit aquatic organisms such as phytoplankton [6]. The organic material is decomposed by decomposer microbes to

become nutrients (N and P) and can be used for microalgae growth [7]. On the other hand, if the organic material is not in accordance with the carrying capacity, it will have a negative impact because it will increase the rate of O₂ reduction and increase the O₂ requirement in the sediment, which will produce reduced compounds, such as NH₃, CH₄, and H₂S [6]. In addition, the effectiveness and amount of nutrients by phytoplankton depends on the availability of NO₃-N, PO₄-P absorption and organic materials, which are influenced by pH, dissolved oxygen, temperature, water transparency, salinity, NO₂-N and NH₃-N [8].

Phytoplankton is microorganisms that live hovering in the oceans, lakes, rivers, and other water bodies, are autotrophic microorganisms that can produce their own food with the help of sunlight [9]. The level of plankton production in an area of water can be used to estimate the potential for shrimp and fish production [10]. As well as providing a nutritional source for shrimp, phytoplankton is also very important in improving water quality [8]. The existence of phytoplankton can be used as a bioindicator of changes in the aquatic environment caused by an imbalance of an ecosystem due to pollution [11]. The high uniformity of plankton with the high number of individuals of each plankton and evenly distributed plankton will result in prime environmental conditions and can increase pond productivity [12]. The fluctuation in air quality parameters can cause a decrease in shrimp

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survival which continues to decrease in production [13]. This research was conducted to analyze the relationship between phytoplankton community structure in the waters and its relationship with the productivity of white shrimp (*Litopenaeus vannamei*) culture, by observing the diversity, density of phytoplankton, water quality during cultivation, and the productivity of the aquaculture pond.

MATERIAL AND METHOD

The research was conducted from 5th January – 5th February 2021. Samples were taken from two *vannamei* ponds in Jatisari Village, Banyuwangi Regency. Identification is carried out in the research location laboratory. We used plankton net, film bottle, water sampler, coolbox, hemocytometer, cover glass, dropper pipette, microscope, identification book, washing bottle, DO meter, refractometer, Secchi disk, and 5L bucket. We also used water samples, 70% alcohol, lugol, distilled water, test kits for NH₄, NO₃, NO₂, PO₄, and pH paper.

Parameters

The main parameters observed were phytoplankton density, diversity index, and performance of cultivation which included productivity, survival rate, FCR (Feed Conversion Ratio), and ADG (Average Daily Growth). The supporting parameters observed during the study were water quality. Water quality parameters observed during the study were temperature, water transparency, dissolved oxygen, pH, ammonia, nitrite, nitrate, and phosphate.

Phytoplankton sampling

The research samples were taken at four points, inlet, outlet, and two ancho shrimp ponds (*Litopenaeus vannamei*). Samples were taken every day at 13.00 WIB (West Indonesian Time). The collection was done using a bucket with a volume of 5 L for each point. The sample water is put into the plankton net, which has been given a 20 mL film bottle, then given four drops of lugol solution to preserve the phytoplankton and ensure that there is no eating process in the film bottle. Samples that have been taken are labeled and placed in the coolbox.

Phytoplankton identification and calculation

Calculation of phytoplankton density (cells. mL⁻¹) using the Big Block calculation formula [14] as follows:

$$\text{Phytoplankton density} = \frac{nA + nB + nC + nD}{4} \times 10^4$$

Description:

nA, nB, nC, nD : Number of of phytoplankton cell in blocks A, B, C, D
4 : Number of blocks counted
10⁴ : Conversion factor mm³ to cm³

The diversity index is calculated using the Shannon-Wiener method with the following formula:

$$H' = - \sum_{i=1}^n pi \ln pi$$

Description:

H' : Shannon-Wiener diversity index
Pi : ni / N
ni : Number of individuals of type i
N : Total number of individuals

Aquaculture Performance Measurement

The formula used to measure the value of SR [15] is:

$$SR = \frac{N_t}{N_0} \times 100\%$$

Description:

SR : Survival (%)
Nt : Amount of harvest
N0 : Total stocking

The Feed Conversion Ratio (FCR) is calculated using the formula according to [15] is:

$$FCR = \frac{F}{Wt + D}$$

Description:

FCR : Feed conversion ratio
F : Weight of feed given (g)
Wt : Biomass at the end of maintenance (g)
W0 : Initial maintenance weight (g)
D : Weight of dead fish (g)

Average Daily Growth (ADG) is calculated using the formula [16]:

$$ADG (g \cdot day^{-1}) = \frac{(Wt - W0)}{t}$$

Description:

ADG : Daily weight gain
Wt : Final weight (g)
W0 : Initial weight (g)
T : Duration of maintenance (days)

Water Chemical Physics Parameters

Dissolved oxygen, temperature, pH, water transparency, salinity were measured every day during the study, while ammonia, nitrite, nitrate, and phosphate were measured every three days. Dissolved oxygen was measured at 06.00, 13.00, and 20.00 WIB (West Indonesian Time), water transparency, temperature, and pH were measured at 06.00 and 13.00 WIB, salinity was measured at 06.00 WIB. Ammonia, nitrate,

nitrite, and phosphate were measured every three days at 06.00 WIB.

RESULT AND DISCUSSION

Phytoplankton Identification

The results obtained in the two ponds, in pond 1, there were six classes with a total of 31 genera consisting of Cyanophyceae (10 genera), Chlorophyceae (9 genera), Bacillariophyceae (7 genera), Dinophyceae (3 genera), Euglenophyceae (1 genus), Cryptophyceae (1 genus). In pond 2, six classes were found, with a total of 31 genera. The classes found in pond 2 are Cyanophyceae (10 genera), Chlorophyceae (8 genera), Bacillariophyceae (8 genera), Dinophyceae (3 genera), Euglenophyceae (1 genus), Cryptophyceae (1 genus). The highest number of genera in both ponds is Cyanophyceae.

Chlorophyceae and Bacillariophyceae are phytoplankton, which are expected in cultivation [17] as natural food and oxygen pponder [18], while Cyanophyceae are types that are avoided for cultivation because they contain toxins that will cause the organism to be susceptible to disease [19]. Bacillariophyceae are often found in the sea in large numbers [18], so that this class is commonly found in vannamei shrimp farming locations.

Phytoplankton Composition and Density

The percentage of phytoplankton composition in pond 1 is known to have three classes that dominate with high values, Cholorophyceae, Cyanophyceae, and Bacillariophyceae, with the percentages that can be seen in Figure 1. The percentage of other classes found in both ponds is below 10%. The dominant phytoplankton classes found during the study were common in the white shrimp (*Litopenaeus vannamei*) cultivation location. The results of the research conducted previously have three dominant classes, which are the same as the results for which the study was conducted [8,18,20]. The phytoplankton that is expected to grow in pond waters is Bacillariophyceae and Chlorophyceae because these two classes can be used as natural food for shrimp other than as an oxygen enhancer in the water column [18].

Bacillariophyceae in both ponds had the same percentage. The genus of the class Bacillariophyceae can dominate in pond waters because of the availability of nutrients essential for growth in the form of ammonia, nitrite, and nitrate due to feeding [21]. Bacillariophyceae is a phytoplankton that can adapt to various

environments and is cosmopolitan [22]. Generally, Bacillariophyceae class has high nutritional value, is easy to digest, and is very good, especially for the survival of shrimp larvae, and this species is preferred by shrimp compared to other classes [8].

The high percentage of Cyanophyceae in both ponds could be due to the high phosphate value in both ponds. Phosphate levels greater than 0.01 mg.L⁻¹ can lead to predominance in waters [18]. If there is a blooming of Cyanophyceae in the waters, it will cause the water to be green, blue, and even black, and it will be less profitable because Cyanophyceae releases toxins that are harmful to shrimp which can cause death before harvest [21].

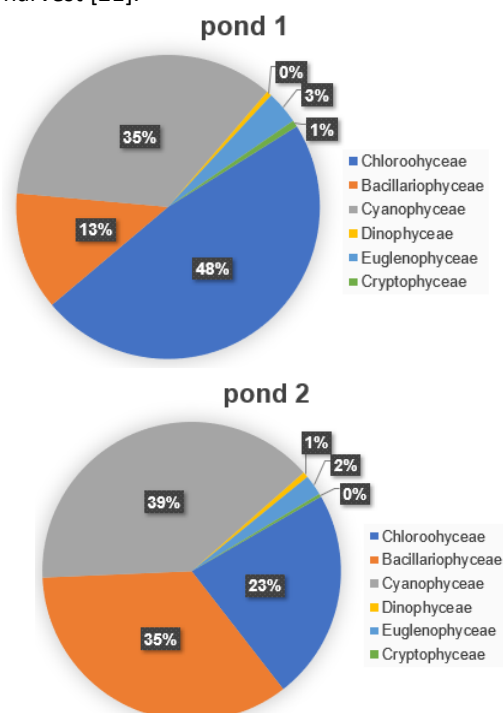


Figure 1. Composition of Phytoplankton

The diversity index value in the study can be seen in Table 1. The research diversity index has an index similar to the study by Umami *et al* [8] and Mansyah *et al* [19] and higher than the study by Hendrajat and Sahrijanna [23]. The index shows that the diversity value is moderate ($1 < H' < 3$), which means that pond water quality is still good to support the development and diversity of phytoplankton [19]. The greater the H' , indicating that the more diverse life in water is [24]. If the diversity index is close to 1 (> 0.5), then the uniformity of organisms in the waters is in a balanced state, and there is no competition for both place and food, but these values indicate unstable biota [23].

The effectiveness and uptake of nutrients by phytoplankton in pond waters depend on the availability and absorption rate of NO₃-N, PO₄-P, and organic matter which is influenced by pH, dissolved oxygen, temperature, water transparency, salinity, NO₂-N, and NH₃-N [25]. Optimal water conditions will spur the absorption rate of nutrients by phytoplankton so that it can improve the structure of the plankton community. The N:P value in both ponds ranged from 1.91-13.79, and the average N:P value in pond 1 was 6.59 and in pond 2 was 7.28. Cyanophyceae were found to be more dominant at low N:P ratios below 10: 1 [26].

Cultivation Performance

Based on Table 2, yields and SR of both ponds have a higher value than the study by Aridin *et al*

[20], the SR value in pond 1 is 92%, with a yield of 5636 kg for a pond area of 2000 m² and in pond 2 is 80%, with a yield of 7688.5 kg for a pond area of 2500 m². The high SR value indicates that the environmental conditions in the water for the production process are quite good [13].

The FCR value for pond 1 is 1.08, and pond 2 is 1.13. The FCR value in this study is greater than Budiardi *et al* [13]. The lower the FCR value, the better condition, which shows that the optimal feeding for cultivation. Generally, the FCR value in vannamei shrimp culture is 1.4-1.8 [27]. In addition, water quality also affects shrimp appetite, which will affect the FCR value. Temperature, salinity, and alkalinity have a close relationship with the FCR value of vannamei shrimp [28].

Table 1. Phytoplankton Genera and Average Density

No	Genus name	Class	Pond 1		Pond 2		
			Average density (cell.mL ⁻¹)	Average genus (%)	Average density (cell.mL ⁻¹)	Average genus (%)	
1	<i>Oocystis</i>		3,666	1.02	4,750	1.85	
2	<i>Nanochloropsis</i>		159,916	44.8	48,333	18.8	
3	<i>Cosmarium</i>		83	0.02	166	0.06	
4	<i>Tetreselmis</i>		416	0.11	0	0	
5	<i>Golenkinia</i>	Chlorophyceae	250	0.07	83	0.03	
6	<i>Closterium</i>		166	0.04	83	0.03	
7	<i>Tetraselmis</i>		0	0	1,000	0.39	
8	<i>Tetraedron</i>		83	0.02	0	0	
9	<i>Dictyosphaerium</i>		83	0.02	166	0.06	
10	<i>Gleocystis</i>		683	1.91	3,833	1.49	
11	<i>Skeletonema</i>			14,750	4.14	31,666	12.36
12	<i>Cyclotella</i>			11,916	3.34	12,750	4.9
13	<i>Nitzschia</i>		Bacillariophyceae	166	0.04	833	0.3
14	<i>Coscinodiscus</i>			666	0.18	666	0.26
15	<i>Navicula</i>	166		0.04	500	0.19	
16	<i>Bidulphia</i>	0		0	83	0.03	
17	<i>Chaetoceras</i>	16,750		4.70	27,000	10.54	
18	<i>Thalassiosira</i>	750		0.21	15,666	6.11	
19	<i>Microcystis</i>			14,750	4.14	23,000	8.9
20	<i>Chroococcus</i>			40,750	11.44	1,4750	5.7
21	<i>Oscillatoria</i>	Cyanophyceae	28,416	7.97	27,166	10.6	
22	<i>Merismopedia</i>		666	0.18	1,000	0.39	
23	<i>Anabaenopsis</i>		916	0.25	916	0.35	
24	<i>Anabaena</i>		30,083	8.44	22,833	8.91	
25	<i>Gleocapsa</i>		666	0.18	1,416	0.55	
26	<i>Pseudanabaena</i>		666	0.18	2,000	0.78	
27	<i>Synechococcus</i>		3,250	0.92	2,500	0.97	
28	<i>Spirulina</i>		2,166	0.60	4750	1.85	
29	<i>Protoperidinium</i>		Dinophyceae	250	0.07	166	0.06
30	<i>Gymnodinium</i>	1,083		0.30	1,166	0.45	
31	<i>Noctiluca</i>	666		0.18	250	0.09	
32	<i>Euglena</i>	Euglenophyceae	2,000	3.55	566	2.2	
33	<i>Criptomonas</i>	Criptophyceae	2,500	0.7	833	0.3	
Total			356166	100	256000	100	
H'			1.7602442		2.0218753		

The ADG value in pond 1 ranged from 0.19-0.48 g.day⁻¹, with an average of 0.31 g.day⁻¹, and in pond 2, it ranged from 0.19-0.55 g.day⁻¹ with an average of 0.357 g.day⁻¹. The highest ADG in the study had a higher value than the study [16]. The lowest ADG value of the two ponds was caused by the adjustment period of the vannamei shrimp from natural feed to artificial feed.

Table 2. Cultivation Performance

No	Indicator	Pond 1	Pond 2
1	Cultivation age (day)	72	73
2	Yields (kg)	5,636	7,688.5
3	Survival rate (%)	92	80
4	FCR	1.08	1.13
5	ADG (gr.day ⁻¹)	0.316	0.357

Water Quality

Water quality during the study can be seen in Table 3. The temperature range during the study was 27.5-31°C. Bacillariophyceae and Chlorophyceae are found in many stable water conditions. The optimum temperature for the growth of phytoplankton from Bacillariophyceae is 30-35°C and Chlorophyceae 20-30°C, Cyanophyceae can tolerate higher temperatures (above 35°C) than the Chlorophyceae and Bacillariophyceae classes [5].

The temperature has an influence on the oxygen consumption, growth, survival rate of shrimp in a cultured environment [29]. In addition, the temperature has a direct influence in controlling the rate of various metabolic processes of microalgae cells [21]. The temperature tolerated by vannamei shrimp [30] ranges from 23-30°C and grows optimally at temperatures of 30°C (for small shrimp, 1g) and 27°C (for large shrimp, 12-18g). Vannamei shrimp can also tolerate temperatures below 15°C and above 33°C, but their growth will be stunted.

Water transparency is a condition that describes the ability of sunlight to penetrate the water layer to a certain depth [31]. The range of water transparency measured during the study in

the two ponds was 20-53 cm. The highest water transparency is in pond 1, which is 48 cm in the morning, and the lowest is 20 cm during the day. The highest water transparency is in pond 2, which is 53 cm during the day and the lowest is 27 cm during the day. The lower the water transparency value, the increased phytoplankton abundance [13], besides that, turbidity or low water transparency can be caused by nutrient content and sludge [31]. Good pond water transparency for shrimp rearing is 35-45 cm [32].

The pH range values obtained in the two ponds were 7.6-8.5. The pH value range of 7.17-8.98 in ponds does not harm cultivation organisms, including plankton [3]. The optimum pH range for cultivation activities is between 7.0-9.0 [33]. The limit of tolerance of organisms to pH varies and is influenced by temperature, dissolved oxygen, alkalinity, the type of organism, and its place of life [33].

Salinity is a reflection of the amount of salt dissolved in water [21]. Vannamei shrimp can tolerate a wide range of salinity, 0.5-45 ppt, can grow well in the range of 7-34 ppt [30], and grow optimally at 25-30 ppt [32]. The diatom class group was able to adapt to salinity up to 90 ppt [12]. So that the salinity obtained during the study is in the optimal range for the growth of shrimp and phytoplankton in water. Salinity during the study ranged from 25-31 ppt.

Oxygen has an important role in the survival of aquatic organisms. The lowest DO (dissolved oxygen) value was in pond 1, 2.9 ppm at night, and the highest was 8.7 ppm during the day. The lowest DO (dissolved oxygen) value was in pond 2, 2.5 ppm at night, and the highest was 9 ppm during the day. Marine species will die if DO levels are below 1.25 mg.L⁻¹ for several hours. DO values between 2.5-3 mg.L⁻¹ result in reduced swimming speed, and DO levels of 5.3-8 mg.L⁻¹ are good for the survival and growth of marine organisms [34]. Plankton can live well at oxygen concentrations of more than 3 mg.L⁻¹ [35].

Table 3. The average value of water quality during study

Parameters	Pond 1			Pond 2			Optimum value	References
	06.00	13.00	20.00	06.00	13.00	20.00		
Temperature (°C)	28.1	29.2	-	29.3	30.45	-	23-30°C	[30]
Water transparency (cm)	31.8	30.4	-	35.4	34.2	-	35-45 cm	[32]
pH	7.7	8.17	-	7.7	8.2	-	7.0-9.0	[33]
Salinity (ppt)	28	27.9	-	28.5	28.3	-	25-30 ppt	[32]
DO (ppm)	3.8	6.4	3.6	4.0	7.1	3.8	5.3-8 ppm	[34]
NH ₄ (mg.L ⁻¹)		1.25			1.3		< 1.7 mg.L ⁻¹	[39]
NO ₂ (mg.L ⁻¹)		6.47			7.2		< 0.05 mg.L ⁻¹	[40]
NO ₃ (mg.L ⁻¹)		14.9			14.8		< 0.5 mg.L ⁻¹	[41]
PO ₄ (mg.L ⁻¹)		2.75			2.7		0.27-5.51 mg.L ⁻¹	[41]

The DO value of the two ponds at night is lower than the optimum value for phytoplankton and vannamei shrimp. The high DO content during the day in both ponds was caused by oxygen intake produced by phytoplankton [18] and waterwheels [34]. The availability of windmills provides dissolved oxygen is always available, and the pond is always in aerobic conditions. It is the response to the decomposing bacteria that accelerate the decomposition process of organic waste into N and P nutrients, which are fixed into $\text{NO}_3\text{-N}$ and $\text{PO}_4\text{-P}$. These conditions can effectively accelerate nutrient uptake of phytoplankton growth [25]. Dissolved oxygen dynamics in aquatic ecosystems are determined by the balance between oxygen production and consumption [36].

In intensive ponds, ammonia is mostly in the form of NH_4^+ , which can be ionized due to the availability of dissolved oxygen, and the N source from NH_4^+ is not toxic to aquatic organisms and can be utilized directly by phytoplankton [25]. NH_4^+ is required for phytoplankton to form protein and cell formation [37]. The range of NH_4^+ values during the study in pond 1 was 0.4-2.5 mg.L^{-1} , and in pond 2, it was 0.2-2.5 mg.L^{-1} . This value was higher than that of the study [38]. Concentrations $<1.7 \text{ mg.L}^{-1}$ are not hazardous for aquaculture activities [39].

Nitrite (NO_2) levels should not exceed 0.05 mg.L^{-1} because they can be toxic to aquatic organisms [40]. Meanwhile, the range in the two ponds was 0.75-11.25 mg.L^{-1} . This value is certainly higher than Effendi [40] and the result is higher than the study of Utojo and Mustafa [25].

Based on measurements during the study, it was known that the nitrate value in pond 1 ranged from 3-25 mg.L^{-1} with an average of 14.8 mg.L^{-1} . In pond 2, nitrate values ranged from 3-40 mg.L^{-1} with an average of 14.9 mg.L^{-1} . The nitrate concentration for vannamei shrimp culture is $<0.5 \text{ mg.L}^{-1}$, while the optimal nitrate level for phytoplankton growth is 3.9-15.6 mg.L^{-1} . If it is lower than 0.114 mg.L^{-1} , nitrates will be a limiting factor for phytoplankton [41]. Nitrate content above 0.5 mg.L^{-1} is feared to cause eutrophication in the waters. So that both ponds are good enough for phytoplankton but too high for vannamei shrimp cultivation.

The optimal phosphate content for phytoplankton growth is in the range 0.27-5.51 mg.L^{-1} [41], while the phosphate content less than 0.02 mg.L^{-1} will be a limiting factor. Based on measurements during the study and additional data from the study location, it was

found that in pond 1, the phosphate values ranged from 2.5-3 mg.L^{-1} with an average of 2.75 mg.L^{-1} . In pond 2, the phosphate values ranged from 2.2-3 mg.L^{-1} with an average of 2.7 mg.L^{-1} , so that the results obtained during the study have an optimal range for cultivation and phytoplankton.

The higher NH_4 , PO_4 , and NO_3 content with the support of high water temperature and water transparency will result in increased chlorophyll biomass content [38] so that the abundance of phytoplankton biomass increases. The process of decomposing organic matter into mineral salts is faster when the pH range is in the alkaline range [32].

CONCLUSION

The performance value of vannamei shrimp culture in this study shows a good value. It can be caused by the optimal aquatic environment so that the shrimp's appetite can be maintained. Phytoplankton stability during the study also affected the cultivation performance value. ADG and FCR values indicate that the shrimp make good use of the feed.

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Identification of Sago-Pulp Amyolytic Bacteria and Its Utilization for Granulated Fertilizer

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Abstract

Previous research stated that bacteria isolated from sago waste from the traditional sago industry in Palopo had the potency to produce amylase. This study aims to confirm the ability of bacteria isolated from sago pulp waste to produce amylase, evaluate the ability of these amyolytic bacteria to produce IAA fix nitrogen, identify selected bacteria, and apply selected bacteria into granules biofertilizers. Bacteria were isolated from sago pulp waste and grown on a 1% starch agar medium. The amyolytic activity was analyzed qualitatively using iodine and quantitatively using the 3,5-Dinitrosalicylic Acid (DNS) method at pH 6. Amyolytic bacteria were analyzed for IAA production using Salkowsky reagent and nitrogen fixation ability through the Serra Kit method. Potential bacteria were identified based on the similarity of the 16S rDNA sequence. The selected bacteria were grown in a 10mL NB medium, and then bacteria were inoculated and air-dried to obtain the granules. The selected bacteria were put into granules and re-isolated using the total plate count (TPC) method. Eight amyolytic bacteria were obtained from sago pulp waste. L1E isolate had the highest amyolytic activity of 1.228 U.mL⁻¹, and L1D isolate had the highest IAA production of 69.8 g.mL⁻¹. Isolate L1E was identified as *Alcaligenes faecalis* with a 99.45% similarity index, and L1D was identified as *Serratia surfactantfaciens* with a 99.09% similarity index. Isolate L2G was identified as *Alcaligenes aquatilis* with a similarity index of 99.8% of the 16S rDNA sequence. The viability of bacteria from granules was 1.41 x 10⁸ CFU.g⁻¹.

Keywords: Amylase enzyme, amyolytic bacteria, granulated fertilizer.

INTRODUCTION

Indonesia has approximately 1.2 million ha of sago plantation producing around 8.4-13.6 million tons of sago annually. Sago becomes the staple food for some areas in Indonesia as it contains high nutrient and calories sources. In South Sulawesi, especially Palopo City, sago becomes the main source of food and income for the local community. The processing of sago remains limited to produce sago flour for basic food. Sago flour can be produced from the extraction of sago stalks. The cycle of sago processing produces solid and wastewater, including the bark of sago, fibrous sago residue (pulp), and wastewater. It is about 26% of sago bark and 14% of sago pith count of the total weight of the sago block. Sago waste remains to have about 65.7% of dissolved starch [1].

The sago waste produced annually ranges between 1,838 – 2,100 tons.year⁻¹ [2]. Sago pulp waste in the sago industry is not handled properly, allowing the accumulation of solid waste and causing acidity in the soil, then polluting the environment. Sago waste can be used as a substrate for bacterial growth and to

be used to produce various commercially useful products. The starch content in sago pulp ranges from 41.7 to 65% [2]. Sago waste can be well hydrolyzed into reducing sugars by bacteria with a degrading activity such as amyolytic bacteria to degrade the starch components. Therefore, the use of potential bacteria to degrade starch is needed to maximize the bioconversion of sago pulp [3].

Biofertilizer is a material containing certain live microbes that function as a plant growth-promoting (PGP) producer, a nitrogen fixer, and a decomposer. One type of bacteria that can be used is amyolytic bacteria. Amyolytic bacteria such as the genus *Bacillus*, *Enterobacter*, and *Klebsiella* are known to have the ability to fix nitrogen [4]. Therefore, this study focused on obtaining amyolytic bacteria from sago pulp in Palopo, South Sulawesi, that can be further used for amylase, IAA production, and nitrogen-fixing, so it will be a biofertilizer agent to solve the sago pulp waste problem.

MATERIAL AND METHOD

A Sampling of Sago Pulp Waste Collection

The sago pulp waste was collected from the traditional industry of sago in Palopo, South Sulawesi, at two locations, location 1 (L1) in Salubulo and location 2 (L2) in Songka (Fig. 1). Both locations were chosen based on the different activities of traditional sago industries,

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which led to the assumption the sago pulp waste differs in its storage time. Sample of sago pith waste was taken from a pile of sago pulp waste at the bottom and near the ground, then stored in a plastic bag in an isotherm box. Measured environmental parameters, such as C/N ratio,

organic compound, and pH, were measured to complete the information of physicochemical conditions in both locations. The organic compound and C/N ratio were analyzed in Soil Laboratory, Faculty of Agriculture, University of Brawijaya.

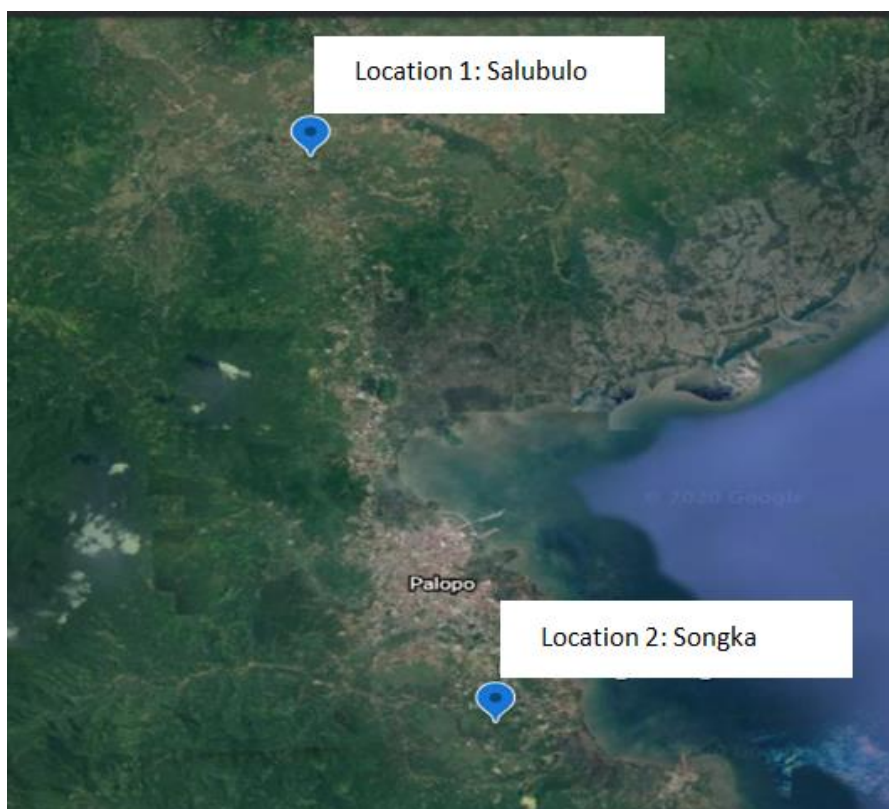


Figure 1. Locations of sago waste sampling (location 1: Salubulo, location 2: Songka)

Isolation and Screening of Amyolytic Bacteria

Isolation of amyolytic bacteria was carried out using starch media (1% soluble starch, 0.2% yeast extract, 0.5% peptone, 0.05% MgSO₄, 0.05% NaCl, 0.015% CaCl₂, and 2% bacteriological agar) with method plate [2,5]. All plates were incubated at 30°C for 48 h. The isolates were screened used iodine 1% by flooding the isolates inoculated in another petri dish, and a clear zone indicated a positive value. The isolated colonies were photographed, then grouped.

Crude Enzyme Production of Selected Bacteria and Screening of Amyolytic Bacteria

Each selected bacteria were inoculated into 25 mL starch broth media then incubated in a shaker incubator at 30°C 120 rpm for 48 h. Five ml of the bacterial culture was taken to observe the optical density at 54 nm, equalize the sample density, taken 1 mL of each, then transferred to 50 mL of new starch broth media, incubated at

37°C 120 rpm. Five mL of the samples were taken at 24, 48, 72, and 92 hours. The optical density (OD) was measured at a wavelength of 540 nm [6]. The production of crude enzyme was obtained by centrifugation of culture medium at 4°C, 10000 rpm for 10 minutes. The supernatant was defined as the crude amylase enzyme then taken to analyze the enzyme activity [7].

Data Collection

Amyolytic Activity Assay

Amylase activity was tested using the DNS method [8]. One mL of crude amylase filtrate from centrifugation (supernatant) was put into a test tube, then added liquid starch media with 1% sago as a source of starch and then incubated at 37°C for 15 min. One g.100mL⁻¹ of starch solution was added to the blank tube (as blank) and then incubated at 15 min, 37°C without added the enzyme. One mL of the DNS reagent was added to each test tube to stop the reaction

after 15 min. The tube was heated to boiling for 5 min, added 1 mL of K-Nartrate solution, cooled with running water for 15 min and 1 mL of distilled water added. Each solution in the test tube was then determined by the color intensity using a spectrophotometer at 540 nm. The absorbance value was plotted with a glucose standard curve. Each test sample was repeated three times for enzyme activity [8,9].

Indole-3-Acetic acid Assay

The amount of IAA produced by amyolytic bacteria was determined by a colorimetric technique using Van Urk Salkowski reagent and Salkowski's method [10]. The isolates were grown in Triptic soy broth and incubated at 28°C for four days at a shaker incubator. Data were obtained from the sampling of 24, 48, 72, and 96 hours. The broth was centrifuged after incubation. The supernatant was reserved, and 1mL was mixed with 2 mL of Salkowski's reagent (2% 0.5 FeCl₃ in 35% HClO₄ solution) and kept in the dark. The optical density (OD) was recorded at 530 nm after 30 min [11].

Nitrogen Fixation Assay

Cultures of each isolate were equalized for age, culture volume, and cell density. Each isolate culture in the logarithmic growth phase was inoculated as much as 5 mL in 50 mL of liquid Nfb without the addition of Bromothymol Blue. The control (media without the addition of inoculum) was also prepared. Controls and test cultures were incubated in a shaker incubator at 120 rpm at 30°C for seven days. The culture was taken 2 mL and centrifuged at 10,000 rpm at 28°C for 15 minutes. The supernatant was taken 1 mL and put into a test tube. The ammonia content of the supernatant was determined based on the Sera Ammonia Test Kit. A change in color indicated the nitrogen-fixing activity of each isolate from clear to green.

Bacteria Identification Based on 16S rDNA

Bacterial chromosomal DNA was extracted using a Quick-DNA™ Fungal/Bacterial Miniprep Kit (ZYMO RESEARCH, USA). The 16S rDNA sequence was amplified by Polymerase Chain Reaction (PCR) using universal primers 27f (5'-AGAGTTTGATCCTGGCTCAG3') and 1492r (5'-GGTTACCTTGTTAACGACTT-3') with PCR program: pre-denaturation at 94°C for 5 min, followed by denaturation at 94°C for 30 min, annealing at 55°C for 30 s, and extension at 72°C for 5 min. The amplicon of 16S rDNA was confirmed by 1.5% agarose gel electrophoresis and visualized

using a UV transilluminator. The amplicon of 16S rDNA was purified and sequenced in First BASE, Malaysia. The 16S rDNA sequences of bacteria were aligned with reference strains from the GenBank database using the MEGA V.6 program, and the phylogeny tree was constructed and inferred according to the Neighbor-joining algorithm and Tamura-Nei model [12].

Hemolytic Activity Assay

Hemolytic activity was assayed to determine phenotypically non-pathogenic bacteria. Bacterial isolates were tested by growing on 5% blood agar media, then incubated at 30°C for 24 hours. Bacterial isolates were observed for alpha hemolysis, beta hemolysis, and gamma hemolysis activities. Isolates with gamma-hemolytic activity (no clear zone was formed around the colony) indicated that the bacterial isolates were not pathogenic [13].

Granulated Fertilizer Production

One oose of pure bacterial isolate was selected into a culture bottle containing 10 mL of sterile NB media and then incubated in an incubator shaker at 150 rpm, temperature 28 C for 24 hours. Bacterial density was measured using a spectrophotometer (OD=0.5, wavelength 470nm) then 2.5 mL (5% v/v) of starter culture was inoculated into a 50 mL sterile NB medium as a production medium. This medium was then incubated in a shaker incubator at a speed of 150 rpm at 28°C for 48 hours.

Sago waste as much as 300 grams (part of the crude fiber removed) then dried at a temperature of 40-45°C until the water content is about 15%. The sago pulps were mixed with bacterial starter culture and molasses (ratio 1:1) until compact and could be clenched, then passed through a 14 mesh sieve and air-dried for 24 hours. The evaluation of the obtained biofertilizer granules was then tested for bacterial viability.

Cell density in compost media was calculated using the TPC (Total Plate Count) method. Cell density was calculated after the granules were successfully formed. A total of 1 g of the granule product was diluted in 9 mL of sterile NaCl, then homogenized and diluted to a dilution level of 10⁻¹⁰, then 100 L was taken and poured into sterile Petri dishes. The cup was then poured with sterile NA medium and moved to form a figure of eight and incubated at 28°C for 24 hours, and the Colony Forming Unit was calculated. Count colonies between 30-300.

RESULT AND DISCUSSION

Amylolytic Bacteria Isolates

The bacteria isolation of amylolytic bacteria from sago pulps waste obtained eight isolates consisting of five isolates from Salubulo (L1A, L1B, L1C, L1D, and L1E) and three isolates Songka (L2F, L2G, and L2H). The amylolytic bacteria in Salubulo had a density of 59×10^5 CFU.g⁻¹ more than 17.35×10^5 CFU.g⁻¹, and there was a significant difference. The difference in bacterial density was influenced by the content of organic matter, especially C-organic and the C/N ratio was significantly higher in the sago pulp samples in Salubulo than in Songka (Table 1).

Table 1. Comparison of the physico-chemical and biological parameters of sago pulp waste in Sabulo and Songka based on T-test

Parameter	Sabulo (Location 1)	Songka (Location 2)
C-organic (%)	38.9±1.22 ^b	31.5±3.25 ^a
N-total (%)	0.2±0.06 ^a	1.2±0.69 ^a
C/N ratio	174±38.19 ^b	26.8±28.69 ^a
Organic compound (%)	67.3±2.10 ^b	54.5±5.62 ^a
pH	5.9±0.66 ^a	4.7±0.35 ^a
Total plate count (10 ⁵ CFU.g ⁻¹)	59±7.46 ^a	17.35±7.98 ^b

Note: The same notation after the values shows that the parameters do not differ between locations (p>0.05)

High organic C content in a substrate causes microorganisms to overgrow, and at a high C/N ratio, nutrients for microorganisms are well met so that the density of microorganisms is higher. In addition, carbon is a source of energy for the growth of microorganisms, while nitrogen plays an important role in the preparation of amino acids [1].

The physico-chemical conditions that most influence the growth of bacteria are the available carbon and nitrogen sources. The low organic C content in sago pulp samples taken from Songka was due to the activity of carbohydrate

fermentation by microorganisms to form organic acids such as acetic acid, pyruvic acid, and lactic acid [14,15].

Amylase Activity in Bacteria

L1E isolate had the highest ability to produce amylase enzyme, as much 1,407 U.mL⁻¹ at 48 and hours incubation, followed by L1B isolate 1.228 U.mL⁻¹, and L2H isolate 1.066 U.mL⁻¹ at 72 hours incubation (Table 2). Amylase is an extracellular enzyme that can hydrolyze α-1,4-glycidic bonds into dextrans in starch and other small molecules that make up glucose. Amylase enzymes can be produced by different species of *Bacteria*, *Archae*, and *Actinomycetes* [16]. Strain bacteria isolated from sago pulp had a good ability to produce amylase enzyme. The best amylolytic bacteria has produced the highest enzyme concentration at 72 hour incubation time. In case, L1E isolate has high amylase activity in 42 hours incubation. It could be caused by the environment such as pH and temperature were capable for the production activity [9].

Identification of Amylolytic Bacterial Species

The phylogenetic identification based on the similarity of the 16S rDNA sequence showed that the isolate L1E was identified as *Alcaligenes faecalis* NC260419C. The 16S rDNA gene sequence is the gold standard marker in the identification of bacteria. The gene with a length of 1500 bp has alternating conserved and variable regions that permit to design universal primers on the conserved regions and to use the variable for taxonomic classification, therefore the universal primers 27f (5'-AGAGTTTGATCCTGGCTCAG3') and 1492r (5'-GGTTACCTGTAAACGACTT-3') were used in this experiment. The technique is used to accurately determine the phylogenic relationship between bacteria with over 100,000 sequences available at an online database [17].

Table 2. The activity of amylolytic bacteria in different incubation time

Isolates	Enzyme activity in different incubation times (U.mL ⁻¹)			
	24 hour	48 hour	72 hour	96hour
L1A	0.19 ± 0.14 ^{Aa}	1.01 ± 1.45 ^{Ab}	0.39±0.03 ^{Ab}	0.23± 0.06 ^{Ab}
L1B	0.81 ± 0.49 ^{Ba}	0.88 ± 0.93 ^{Bb}	1.23±0.44 ^{Bab}	0.23± 0.14 ^{Bab}
L1C	0.19 ± 0.07 ^{Aa}	0.51 ± 0.08 ^{Ab}	0.26±0.06 ^{Ab}	0.31 ± 0.29 ^{Aab}
L1D	0.35 ± 0.14 ^{ABa}	0.96±0.04 ^{ABb}	0.54±0.25 ^{ABab}	0.49 ± 0.14 ^{ABab}
L1E	0.56± 0.52 ^{ABa}	1.41±0.87 ^{ABb}	1.3±0.025 ^{ABab}	0.65 ± 0.55 ^{ABab}
L2F	0.23±0.06 ^{ABa}	0.456±0.30 ^{ABb}	0.79±0.18 ^{ABab}	0.53 ± 0.05 ^{ABab}
L2G	0.26 ± 0.14 ^{Aa}	0.67 ± 0.54 ^{Ab}	0.35±0.02 ^{Ab}	0.23 ± 0.07 ^{Aab}
L2H	0.37± 0.07 ^{ABa}	0.80 ± 0.08 ^{ABb}	1.07±0.81 ^{ABab}	0.74 ± 0.07 ^{ABab}

Note: Amylase enzyme activity using Tukey's test. The same notation after the values (the capital letters for the same isolate at different times, lowercase letters for different isolates at the same time) shows that the parameters do not differ between locations (p>0.05).

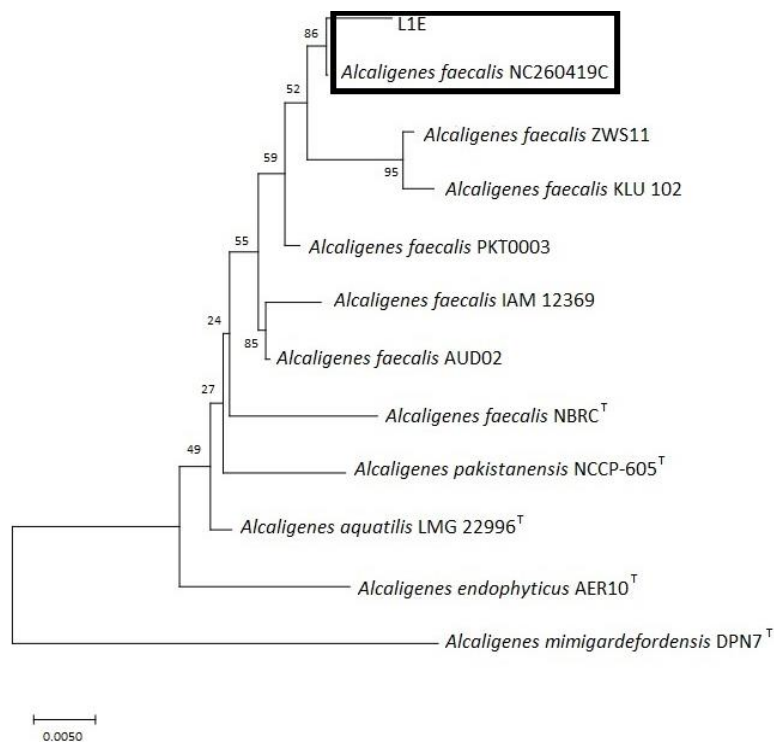


Figure 2. Phylogeny tree showing the relationship between L1E isolates and comparison bacterial strains based on 16S rDNA sequences.

These isolated bacteria had the highest ability to produce amylase enzymes. The results of the 16S rDNA sequence analysis showed that the relationship between L1E isolates and *Alcaligenes faecalis* NC260419C was 99.85% (Fig. 2).

Research on the ability of *Alcaligenes faecalis* bacteria to produce amylase enzymes is still very limited. The results of previous studies showed that the bacterial species *Alcaligenes faecalis* isolated from Taptani hot springs, Odisha, had the ability to produce an amylase enzyme of 4.125U.mL⁻¹ at 84 hours of incubation [18]. The isolate of *Alcaligenes faecalis* bacteria isolated from soil had a maximum amylase enzyme activity at 48 hours of incubation, which was 663.67 U.mL⁻¹ [19].

IAA Hormone Production

L1D isolate had the highest ability to produce IAA hormone, which was 69.83 g.mL⁻¹ at 24 hours incubation, followed by L1E isolate 58.91 g.mL⁻¹ at the same time and L2F isolate 54.45 g.mL⁻¹ at 72 hours incubation (Table 3). Indole-3-Acetic Acid (IAA) is a crucial phytohormone produced by plant growth-inducing bacterial strains. The IAA hormone can increase nutrient absorption activity and better root growth so that it can trigger plant growth [20,21]. The bacteria that produce IAA are *Aeromonas*, *Bacillus*,

Azotobacter, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Microbacterium*, *Sphingomonas*, *Mycobacterium*, and *Rhizobium* [22].

The L1D isolate with the highest ability to produce IAA was *Serratia surfactantfaciens* XY1011, with a similarity index of 99.8% (Fig. 3). *Serratia* sp. is a candidate for the growth-promoting agent in plants because it can produce IAA and break down phosphate. The results of previous studies stated that the isolate could produce 123.2 g.mL⁻¹ IAA after 144 hours of incubation [23]. *Serratia surfactantfaciens* is a group of gram-negative bacteria and members of the Enterobacteriaceae family. These bacteria are often found in water, soil, insects, and plant surfaces [24].

Serratia is associated with plants as endophytic bacteria and free-living species in the rhizosphere. Many *Serratia* species have the ability to increase plant growth, one of which is because it has the ability to produce IAA and also as a biological control agent in pathogenic fungi that spread in soil that infects various plants such as *Erwinia tracheiphila*, *Pseudomonas syringae* pv. *Lachrymans*, and *Fusarium oxysporum* [25]. The bacterial isolates that trigger plant growth are *Serratia* sp. ZoB14 It also protects ginger plants from the pathogenic fungus *Pythium myriotylum* [26].

Table 3 The IAA production in different incubation times

Isolates	IAA production in different incubation times (g.mL ⁻¹)			
	24 hours	48 hours	72 hours	96 hours
L1A	34.08 ± 3.54 ^{hijk}	36.97 ± 10.42 ^{ijkl}	47.04 ± 0.47 ^{lmn}	34.18 ± 1.59 ^{hijk}
L1B	19.15 ± 0.98 ^{cdef}	24.83 ± 0.67 ^{efgh}	22.00 ± 1.35 ^{efg}	17.89 ± 0.37 ^{bcdef}
L1C	8.16 ± 0.99 ^{abcd}	14.08 ± 5.19 ^{abcde}	19.66 ± 0.77 ^{def}	5.47 ± 1.08 ^a
L1D	69.83 ± 9.68 ^p	44.18 ± 1.37 ^{klmn}	24.08 ± 1.41 ^{efgh}	17.14 ± 1.70 ^{bcdef}
L1E	58.91 ± 3.46 ^{op}	51.15 ± 5.78 ^{mno}	51.77 ± 6.69 ^{mno}	28.13 ± 1.89 ^{ghij}
L2F	32.68 ± 4.95 ^{ghijk}	54.45 ± 4.92 ^{no}	42.28 ± 2.18 ^{klm}	22.11 ± 2.32 ^{efg}
L2G	35.03 ± 0.67 ^{ijkl}	38.02 ± 1.49 ^{hijk}	35.54 ± 0.82 ^{bcdef}	27.11 ± 2.26 ^{ghij}
L2H	41.02 ± 2.56 ^{klm}	33.09 ± 2.41 ^{ghijk}	25.51 ± 7.23 ^{efghi}	9.55 ± 0.56 ^{abcd}

Note: The different notation after the values indicates a significant difference between isolates and between incubation times (p<0.05)

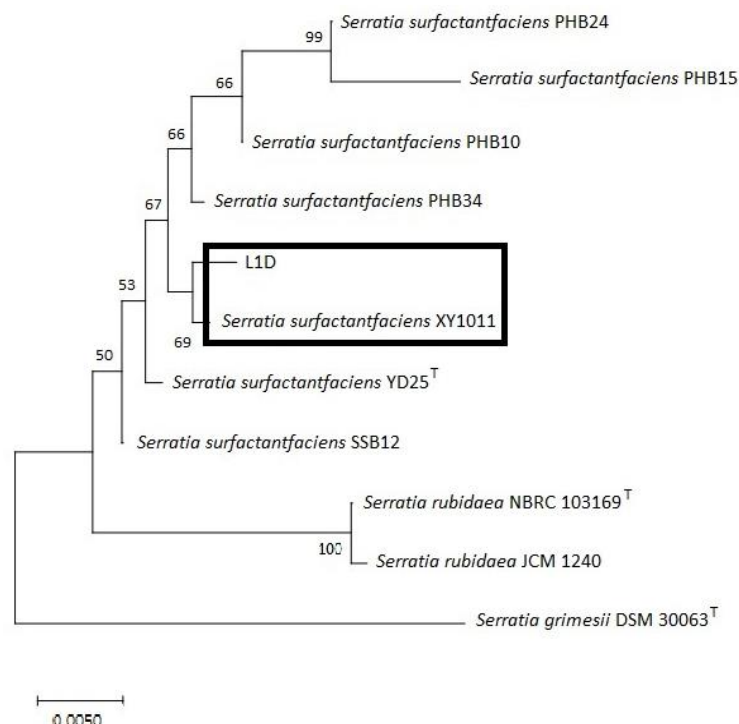


Figure 3. Phylogeny tree showing the relationship between L1D isolates and comparison bacterial strains based on 16S rDNA sequences

Isolates of *Serratia* sp. 5D and RTL100 isolated from root nodules showed the ability to increase yields by 25.55% and 30.85% on fertile soils in irrigated areas and nutrient-deficient soils in rainfed areas when compared to negative controls. These results indicate that the isolates of *Serratia* sp. 5D and RTL100 can function as effective inoculants in increasing nutrient-deficient soil fertility [27].

Nitrogen Fixing Assay

The semi-quantitative N₂ fixation potential test results using the Sera Ammonium Test Kit are presented in Table 4. There were six isolates from a total of eight isolates that could produce ammonia with a concentration of 0.1-1 mg.L⁻¹. The test results showed that isolates L1A, L1B,

L1C, L1D, and L1E could fix N of 0.25mg.L⁻¹. Meanwhile, isolates L2F and L2H could not fix N. Nitrogen-fixing bacteria convert N₂ in the air into ammonia using nitrogenase. The nitrogenase is very sensitive to oxygen; the oxygen concentration as present in the atmosphere (21%) will inhibit the work of enzymes in reducing N₂ becomes NH₃⁺. On the other hand, nitrogen fixation requires the energy of ATP to be synthesized. Through the process of oxidation, especially phosphorylation, which means it requires oxygen. To overcome the opposing needs, leghemoglobin plays a role in helping by binding, transferring, and providing oxygen in the respiration process [28]. Based on the criteria for the highest ammonia production capability, L2G was selected for identification.

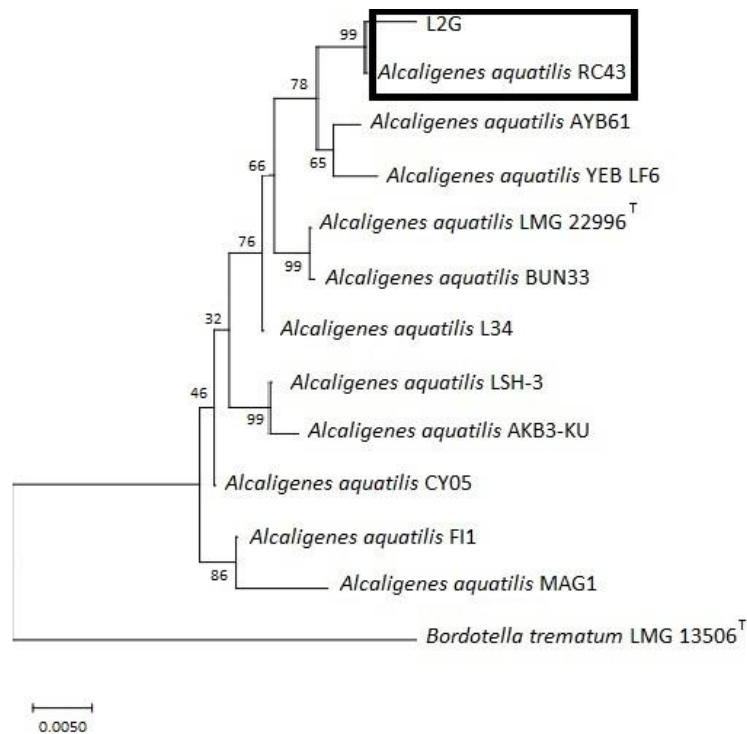


Figure 4. Phylogeny tree showing the relationship between L2G isolates and comparison bacterial strains based on 16S rDNA sequences

Table 4. Semi-quantitative potential assay for nitrogen fixation of amyolytic bacterial

Isolate	Ammonia Production
L1A	++
L1B	++
L1C	++
L1D	++
L1E	++
L2F	+
L2G	+++
L2H	+

Note: +++ = > 1 mg.L⁻¹ dark green color, ++ = 0.5 – 1 mg.L⁻¹ green color, + = 0.1 – 0.4 mg.L⁻¹ light green color, = < 0.1 mg.L⁻¹ yellow color; accumulation of ammonia in bacteria culture

Identification of Nitrogen-fixing Bacteria

The isolate that could fix nitrogen (L2G isolate) was known as the bacterium *Alcaligenes aquatilis* RC43 with a similarity index of 99,8% (Fig. 4.) *Alcaligenes aquatilis* bacteria can live and grow in extreme environments. In addition, these bacteria can grow in an environment polluted by gasoline and its derivatives because it has the ability to fix nitrogen [20].

Hemolysis Assay

The result of the hemolytic activity test of the three potential isolates (*Alcaligenes faecalis*, *Serratia surfactantfaciens*, and *A. aquatilis*), there was one non-pathogenic isolate, namely

Alcaligenes aquatilis isolate with no hemolysis and no changes or no reaction on blood agar after 24 hours of incubation. *Alcaligenes faecalis* and *Serratia surfactantfaciens* isolates were indicated to have beta hemolysis activity with changes in red blood cells to become apparent. Based on this, one isolate was suspected to be non-pathogenic, and two isolates were pathogenic (Table 5).

Table 5. Pathogen confirmation test on selected isolates

Isolates	Hemolytic activity		
	β-hemolytic	α-hemolytic	γ-hemolytic
L1D	+	-	-
L1E	+	-	-
L2G	-	-	+

Note: the value (+) indicates the isolate has the activity, the value (-) indicates the isolate does not have the activity. L2G isolate was chosen as non-pathogenic bacteria

Granulation

The sago pulps were inoculated with L2G isolate, and then the granulation process was carried out. The granules formed were then seen for their bacterial density using the Total Plate Count (TPC) method. TPC results from granulation samples obtained a bacterial density of 1.41 x 10⁸ CFU.g⁻¹. According to the regulation of the minister of agriculture no. 28/Ministry of

Agriculture/SR.103/S/2009 about biofertilizers and soil improvers, the microorganism formula to be used as the starter of biofertilizer were 10^5 CFU.mL⁻¹ [29].



Figure 5. Granules from sago pulp inoculated with L2G isolates

CONCLUSION

Identification using 27f and 1492r primers showed that L1E isolate was identified as *Alcaligenes faecalis*, and L1D isolate was identified as *Serratia surfactantfaciens*. Both L1E and L1D, respectively, have high amylolytic activity and produce high IAA hormones. The L2G isolate was identified as *Alcaligenes aquatilis* RC43 with an ability to fix nitrogen. *Alcaligenes aquatilis* bacteria showed indications of gamma hemolysis, so it assumed that it is safe to use as fertilizer. The results of TPC of granulated fertilizer showed living bacteria numbers of 1.41×10^8 CFU.g⁻¹, where this number met the standard requirements for biological fertilizers.

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Phytoplankton and Its Relationship to White Leg Shrimp (*Litopenaeus vannamei*) Culture Productivity in Alasbulu, Banyuwangi

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Abstract

Shrimp culture is related to phytoplankton's existence as a primary producer and water quality. Aside from their function as natural feed, phytoplankton has a role in maintaining the stability of the pond ecosystem. It is indicated by the high abundance and diversity of phytoplankton, as well as suitable water quality to support the productivity of shrimp culture. This research aimed to determine the correlation between phytoplankton structure community, water quality parameters, and shrimp productivity. This research used a descriptive method. This research was conducted in shrimp culture intensive system Alasbulu Village, Wongsorejo District, Banyuwangi on February-March 2020. The parameters observed are diversity and abundance of phytoplankton, water quality parameters, and production performance in each pond. Based on the results, six phytoplankton classes were identified: Bacillariophyceae (10 genera), Cyanophyceae (8 genera), Chlorophyceae (5 genera), Dinophyceae (2 genera), Euglenophyceae (1 genus), and Cryptophyceae (1 genus). Chlorophyceae dominated both ponds, followed by Cyanophyceae. Diversity index values on ponds 1 and ponds 2 were 1.39 and 1.50, respectively. Productivity of both ponds were 1.8 kg.m⁻² and 1.4 kg.m⁻²; FCR (Feed Conversion Ratio) 1.1 and 1.3; ADG (Average Daily Growth) 0.3 g.day⁻¹ and 0.25 g.day⁻¹. It can be concluded from this research that high density of Chlorophyceae in phytoplankton community is one of the main causes that supported shrimp cultivation.

Keywords: Banyuwangi, *Litopenaeus vannamei*, phytoplankton, productivity.

INTRODUCTION

Aquaculture production from crustaceans or freshwater prawns had a percentage of 29.4% in 2010 and 70.6% for seawater commodities. The production of seawater commodities is dominated by the vannamei shrimp species (*Litopenaeus vannamei*), of which 77% is produced in Asian countries, including Indonesia [1]. Vannamei shrimp is one of the many shrimp species with high economic value and is an alternative type of shrimp that can be cultivated in Indonesia, aside from tiger shrimp (*Penaeus monodon*) and white shrimp (*Penaeus merguensis*). Vannamei shrimp is a superior shrimp commodity that began to be cultivated in Indonesia in 2002 [2].

Vannamei shrimp has various advantages in the cultivation aspect, such as high appetite, good market share, both nationally and internationally. It also has more resistance to disease infection compared to other shrimp species [3]. Intensive vannamei shrimp culture is inseparable from environmental aspects, such as the presence of phytoplankton as primary productivity and water quality. The interaction

form of biological, physical, and chemical conditions of the waters will be determined through interrelated parameters. These parameters will affect primary productivity in the water. Aquatic ecosystems with low biodiversity are unstable and vulnerable to the influence of external pressures compared to ecosystems with high biodiversity. In this case, phytoplankton biodiversity needs to be controlled in order to have an ecosystem that remains in a stable condition [4].

Various biological agents in the aquaculture ecosystem can be used as a determinant of water quality, one of which is the presence of certain phytoplankton groups [5]. Phytoplankton act as a buffer for water quality by increasing dissolved oxygen content through photosynthesis and absorbing toxic substances, such as dissolved ammonia. In addition to functioning as natural food, phytoplankton also functions to maintain the stability of the pond ecosystem [6]. The effectiveness and magnitude of nutrient uptake by phytoplankton in pond water is strongly influenced by the availability of organic matter and other nutrient elements, which are influenced by dissolved oxygen, brightness, salinity, temperature, nitrite, nitrate, phosphate, and ammonia [7].

The environmental stability of the pond water is characterized by high diversity of phytoplankton, a high density of beneficial

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phytoplankton species, and suitable water quality parameters to support the growth of vannamei shrimp. Based on this background, a study was conducted to analyze the presence of phytoplankton in vannamei shrimp (*Litopenaeus vannamei*) ponds and their relationship to cultivation productivity.

MATERIAL AND METHOD

The type of method conducted in this study was descriptive qualitative. This study was carried out from February 16th to March 14th, 2020, in the vannamei shrimp culture pond, located in Alasbulu Village, Wongsorejo District, Banyuwangi, East Java. Sampling was carried out in two ponds (pond 1 and pond 2). The data collected in this study include phytoplankton community which consisted of composition, density, and diversity; cultivation performance which consisted of productivity, Survival Rate (SR), Feed Conversion Ratio (FCR), and Average Daily Growth (ADG); water quality parameters which consisted of temperature, brightness, dissolved oxygen (DO), pH, nitrate (NO₃), nitrite (NO₂), total ammonia-nitrogen (TAN), and phosphate.

Data Collection

The water quality observed consists of physical and chemical parameters. Physical parameters include water temperature and brightness measurements carried out every day at 6 am and 1 pm (West Indonesian Time-WIB). The chemical parameter of DO was measured at 6 pm, 1 pm, and 9 pm. Salinity was measured at 6 pm, while pH was measured at 6 am and 1 pm. Nitrite (NO₂), nitrate (NO₃), TAN, and phosphate were measured three days a week.

Phytoplankton samples were obtained by taking water samples daily in pond 1 and 2 at 6 am. Water samples were taken using a plankton net and collected in a film bottle. Three drops of lugol 1% were added to lessen the movement of phytoplankton. Water samples were taken and dripped onto the hemocytometer surface, which had been closed with a cover glass through the well line. The identification was conducted with Microscope Olympus in 400 times magnification. The density of phytoplankton was measured using the Big Block method, and the diversity index was calculated using the Shannon-Wiener formula.

The data of vannamei shrimp productivity was collected at the end of the cultivation period. The survival rate values were obtained by collecting data of stocking density numbers and

total individual numbers at the end of the rearing period. The ADG values were obtained by calculating both the final and initial weight of vannamei shrimp and comparing them with the rearing period. The FCR values were calculated based on the feed given during cultivation.

RESULT AND DISCUSSION

Phytoplankton Identification

The phytoplankton identified in this study is presented in Table 1. The phytoplankton found in pond 1 and pond 2 consisted of 6 classes with a total of 27 genera. The classes were Bacillariophyceae (10 genera), Cyanophyceae (8 genera), Chlorophyceae (5 genera), Dinophyceae (2 genera), Euglenophyceae (1 genus), and Cryptophyceae (1 genus).

Table 1. Phytoplankton Identified

Class	Genus	Pond 1	Pond 2
Chlorophyceae	<i>Chlorella</i>	35,000	28,393
	<i>Cosmarium</i>	-	2,500
	<i>Oocystis</i>	4,500	6,250
	<i>Golenkinia</i>	3,333	2,500
	<i>Nannochloropsis</i>	50,000	12,500
Bacillario- phyceae	<i>Skeletonema</i>	3,125	3,750
	<i>Cyclotella</i>	16,750	10,000
	<i>Nitzschia</i>	3,750	5,000
	<i>Coscinodiscus</i>	8,333	8,611
	<i>Thalassiosira</i>	-	4,500
	<i>Navicula</i>	3,333	4,167
	<i>Amphiphora</i>	-	2,500
	<i>Bidduphia</i>	2,500	3,214
	<i>Chaetoceros</i>	8,000	8,594
	<i>Amphora</i>	2,500	2,917
Cyanophyceae	<i>Microcystis</i>	8,056	11,071
	<i>Chroococcus</i>	6,250	11,250
	<i>Oscillatoria</i>	13,854	13,182
	<i>Anabaena</i>	8,750	9,500
	<i>Anabaenopsis</i>	4,000	2,500
	<i>Spirulina</i>	4,167	2,500
	<i>Synechococcus</i>	6,667	4,000
<i>Pseudoanabaena</i>	10,000	3,750	
Dinophyceae	<i>Protoperidinium</i>	7,500	4,643
	<i>Gymnodinium</i>	2,500	4,167
Euglenophyceae	<i>Euglena</i>	4,821	4,531
Cryptophyceae	<i>Crypromonas</i>	8,750	7,813
Total		226,439	155,909
Diversity Index (H')		1.39	1.50

The results showed that phytoplankton genera obtained from both ponds were not much different, but pond 2 showed more variation compared to pond 1. Some of the genera that were only found in pond 2 were *Cosmarium*, *Thalassiosira*, and *Amphiphora*. The most genera found in this study were the Bacillariophyceae group. Bacillariophyceae has the ability to adapt to changes in water quality surrounding and has a wide distribution.

Water Quality

Differences in the composition of phytoplankton can be caused by water quality parameters in the pond [8]. Phytoplankton composition is influenced by various factors such as characteristics of the aquatic environment, physical parameters, and chemical parameters [9]. In this study, pond 2 showed a more diverse type of phytoplankton compared to pond 1. It could be attributed to the N:P ratio (Table 2.), where pond 2 showed a higher N:P ratio (0.79 – 32.6) compared to pond 1 (0.5 – 28.7). There is a strong positive correlation between the concentration of nutrients such as N and P with the potential emergence of phytoplankton at the cultivation site. The greater the ratio of N:P, the types of phytoplankton that appear will be more diverse, and their density will tend to increase [10].

Table 2. Water quality parameters

Parameters	Pond 1	Pond 2	Opt. Reference
DO (mg.L ⁻¹)	3.95-11.85	3.68-9.4	>5 [25]
Temp (°C)	27.8-32.5	27.5-33	25-32 [26]
pH	7.1-8.4	6.8-8.3	7-8.5 [22]
Sal (g.L ⁻¹)	19-24	22-30	10-30 [27]
WT (cm)	30-37	25-37	20-40 [28]
NO ₂ (mg.L ⁻¹)	0-1.92	0-2.25	0.1-1 [29]
NO ₃ (mg.L ⁻¹)	0-10	0-10	0.4-0.8 [30]
TAN (mg.L ⁻¹)	0.1-2.25	0-2.85	<1.7 [31]
PO ₄ (mg.L ⁻¹)	0-1.7	0.25-1.0	<0.2 [32]
N:P Ratio	0.5-28.7	0.79-32.6	16:1 [33]

Based on the results obtained, it was shown that the Chlorophyceae group dominated with a percentage of 51% and 44% in pond 1 and 2, respectively. The pH obtained during this study in both ponds ranged from 7.4 – 8.4, where the pH range was optimum for the growth of the Chlorophyceae group. It is known that Chlorophyceae can grow well in the pH range of 7.35 – 8.84 [11].

Phytoplankton Diversity

Based on the observation, it is shown that *Chlorella* dominated the Chlorophyceae group in both ponds. Salinity is one of the water quality parameters that play an important role in the growth and metabolism of *Chlorella*. The population of *Chlorella* will increase along with salinity. *Chlorella* can live in an environment with a salinity value of up to 50 g.L⁻¹ [12]. At the research site, the water used in the cultivation process has a relatively high salinity value, up to 30 g.L⁻¹. It can be one of the reasons for *Chlorella* dominance in both ponds. *Chlorella* can improve the growth performance of shrimp in cultivation

if consumed as much as 3% of body weight every day [13].

The diversity index (H') values obtained from pond 1 and 2 are above 1 (1<H'<3), which indicates that the level of phytoplankton diversity is average, with moderate community stability [14]. The moderate level of phytoplankton diversity obtained in this study is related to water quality conditions, where there are several parameters with values that exceed the threshold in the water, namely TAN (2.85 mg.L⁻¹), nitrate (10 mg.L⁻¹), nitrite (2.25 mg.L⁻¹) and phosphate (1.7 mg.L⁻¹). In addition, the category of moderate water stability is supported by data on the composition of phytoplankton where there is a group that tends to dominate in both ponds, namely Chlorophyceae.

Cultivation Productivity

The results regarding vannamei shrimp (*Litopenaeus vannamei*) culture production in pond 1 and 2 with indicators such as day of culture, yield, survival rate, food conversion ratio, and average daily growth are presented in Table 3. Pond 1 has a higher production yield compared to pond 2 based on the ratio between harvest tonnage (1.8 kg.m⁻²) and pond area (1.4 kg.m⁻²). The high and low production of vannamei shrimp culture depends on various supporting factors, namely growth rate, food that consumed, and density of seed stocking that carried out at the beginning of the cultivation period [15]. This statement is in accordance with the results of this study, where pond 1 has an ADG value of 0.3 g.day⁻¹ with a stocking density of 208 ind.m⁻² so that the productivity results in pond 1 are higher than pond 2.

The high yield value in pond 1 can be associated with phytoplankton density, where pond 1 has a higher value compared to pond 2. The correlation between yield and phytoplankton density in both ponds showed in Fig. 1. Moreover, the amount of production is assumed to be related to the dominance of Chlorophyceae, where pond 1 has a higher percentage of Chlorophyceae density compared to pond 2, which is 51% with a percentage of the genus *Chlorella* at 44.6%. The productivity of vannamei shrimp in Chlorophyceae-dominated pond is relatively higher compared to ponds that are dominated by other phytoplankton groups [16]. One of the genera of Chlorophyceae, namely *Chlorella*, has an omega-3 fatty acid content of 5.52%, which is relatively higher compared to other phytoplankton genera.

Unsaturated fatty acids are good for the growth of organisms so that they can support increasing cultivation productivity [17]. The availability of *Chlorella* as a natural feed can improve the growth performance of shrimp. *Chlorella* contains 80% of protein, eight essential amino acids, vitamins, carbohydrates, fiber, chlorophyll, enzymes, minerals that are useful in supporting growth performance, and the antioxidant compound lutein, which can prevent tissue damage [18].

Table 3. Cultivation Productivity

No	Indicator	Pond 1	Pond 2
1	Pond Area (m ²)	3,514	3,595
2	Stock. Density (ind)	700,408	697,025
3	Day of culture (days)	55	55
4	Yield (kg.m ⁻²)	1.8	1.4
5	SR (%)	80	85
6	FCR	1.1	1.3
7	ADG (g.day ⁻¹)	0.3	0.25

The survival rate value in pond 2 (85%) is higher than pond 1 (80%). The high survival rate in pond 2 is assumed to be influenced by the high diversity index value obtained. Ecologically, phytoplankton has an important function as a primary producer that can be used as an indicator of water fertility. In addition, the biodiversity of phytoplankton shows the level of water stability. The more diverse types of phytoplankton that grow, the more stable the water will be so that it is beneficial for aquaculture activities. Stable water conditions, which include water quality and primary productivity, will support shrimp growth [19].

The lower survival rate obtained in pond 1 can be caused by the high density of antagonistic phytoplankton, one of which is Cyanophyceae. The average total density of Cyanophyceae in pond 1 was 61.744 cells m.L⁻¹, while pond 2 was 57.753 cells m.L⁻¹. Cyanophyceae is a phytoplankton that is detrimental because because of their ability to produce secondary metabolites in the form of toxins that are harmful to shrimp. Cyanophyceae can reduce water quality which will affect the decline in shrimp production. Cyanophyceae can produce various toxins such as microcystins (MCYs), anatoxin-a, anatoxin-a(s), cylindrospermopsin (CYN), and saxitoxin (STX). These toxins have specific target organs, causing organ damage to mortality if present in the body of aquatic organisms at high levels [20]. Besides their ability to produce toxins, Cyanophyceae is a group of

phytoplankton that has lower nutrient content than other phytoplankton groups [21].

The food conversion ratio (FCR) value in pond 1 is 1.1, while in pond 2 is 1.3. Both FCR values are classified as good for cultivation activities. In vannamei shrimp culture, generally, the FCR value ranges between 1.4 – 1.8 [22]. FCR is a ratio between the amount of feed consumed and the weight gain of shrimp. High FCR indicates that more feed is not converted into shrimp biomass. In addition, high FCR also indicates that the treatment given is increasingly ineffective and inefficient [23]. In this study, it was found that the FCR value of pond 1 was lower compared to pond 2. The results indicated that shrimp in pond 1 had a better feed utilization efficiency compared to pond 2. Allegedly, this is supported by the role of natural feed availability in the pond, which can help to reduce the need for artificial feed. Chlorophyceae and Bacillariophyceae are phytoplankton groups that have high nutrient content, so that they are beneficial for shrimp growth. The average total density of Chlorophyceae and Bacillariophyceae in pond 1 was higher than in pond 2. It shows that phytoplankton has the ability to support the fulfillment of nutrients in shrimp.

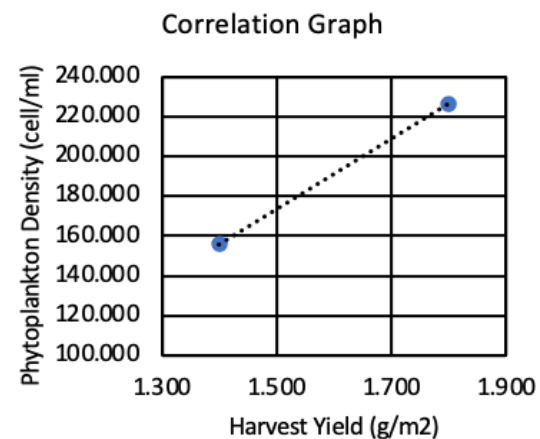


Figure 1. Correlation between total phytoplankton density and harvest yield in linear regression graph.

Average daily growth (ADG) results in pond 1 is 0.3 g day⁻¹, while in pond 2 is 0.25 g day⁻¹. Cultivated organisms in ponds with low stocking densities showed a greater body weight gain. There is less competition in utilizing phytoplankton as natural food in ponds with lower stocking densities [24]. This statement contradicts the result of this study, where pond 2 has a lower ADG value compared to pond 1, but the stocking density in pond 2 (697,025 ind) is lower than pond 1 (700,408 ind). It can be

attributed to the difference in phytoplankton density, where the average total phytoplankton density in pond 1 was higher than pond 2. Based on these results, it can be concluded that although the stocking density in pond 1 is higher than in pond 2, the need for natural feed for shrimp growth in pond 1 is still fulfilled with the abundance of phytoplankton. It shows that shrimp will be able to grow optimally when the need for nutrients is fulfilled.

CONCLUSION

The high and low values of physical and chemical water quality parameters are correlated to the growth of phytoplankton and the survival of vaname shrimp. The phytoplankton found in pond 1 and pond 2 consisted of six classes with a total of 27 genera. The diversity index is in the category of average or moderate water stability. Water quality parameters of temperature, brightness, DO, salinity, pH were in the optimal range, while TAN, nitrite, nitrate, and phosphate exceeded the threshold of the optimal range for cultivation. The productivity of vannamei shrimp culture in pond 1 was higher compared to pond 2 based on the harvest yield, ADG, and FCR values obtained. The high phytoplankton density and dominance of Chlorophyceae that occurred is suspected to be the main cause of the high productivity of cultivation.

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Effect of Prebiotic and Probiotic Fish Feed on Physical, Chemical and Biological Quality of Feed

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Abstract

The limitations of fish in digesting food depend on the presence of enzymes protease, amylase, and lipase that react with substrates in the digestive channel of fish. Supplementation methods can be performed to increase fish growth by adding prebiotics and probiotics to the feed. This study aims to determine the influence of the difference in the length of time fermented prebiotic feed (sweet potato extract) and probiotics (*Bacillus megaterium*) on the quality of feed. The results of the physical analysis of fish feed showed the color looks brown, fishy smell, texture, and general conditions of feed seemed completely normal. Chemical analysis showed that the proteins were best improved in B3 treatment, with the period of fermentation approximately 72 hours. Biological analysis suggests that the longer the fermentation time taken, the higher the abundance of bacteria obtained.

Keywords: *B. megaterium*, chemistry and biology of fish feed, physical analysis, sweet potatoes (*Ipomoea batatas L*)

INTRODUCTION

Certainly, there are obstacles in doing cultivation activities, one of them is the decrease in feed quality caused by the limitations of fish in digesting food depending on the presence of enzymes that react with substrates in the fish digestive system. Therefore, additional ingredients are needed to increase the fish growth and the feed efficiency which are added into feed additives in order to minimize the production costs [1]. Normally, only 20-25% of protein is consumed by fish in intensive cultivation systems [2].

The limitations of fish in digesting food depend on the presence of enzymes protease, amylase, and lipase that react with substrates in the fish digestive system. Supplementation methods are able to be applied in order to increase fish growth by adding prebiotics and probiotics to the feed. The addition of prebiotics and probiotics into the feed with the process of fermentation may increase the quality of fish feed as the microorganisms assist in breakdown the difficult-to-digest substance into smaller pieces [3].

Prebiotics are generally carbohydrate compounds formed as oligosaccharides (oligofructose) and dietary fiber (inulin) [4]. Oligosaccharides are generally found in grains, nuts, and tubers, such as sweet potatoes. Oligosaccharides contained in sweet potatoes (*Ipomoea batatas L*) are maltotriose, raffinose, and oligofructose [5]. Meanwhile,

probiotics in aquaculture have several advantages, such as improving the growth physically, contributing enzymes into nutrition, inhibiting the colonization of pathogenic bacteria in the gastrointestinal tract, modulating the intestinal microbiota, and improving the hematological and immune response [6]. Bacterium derived from the group of *Bacillus* spp. is *Bacillus megaterium* bacteria that are used as probiotics. Thus, the purpose of this study is to figure out the effect of the difference in the length of duration in making fermentation prebiotic feed (sweet potato extract) and probiotics (*Bacillus megaterium*) on the quality of feed.

MATERIAL AND METHOD

This research was conducted at the Laboratory of Technology of Fisheries Products Division of Food Safety, Faculty of Fisheries and Marine Sciences, Brawijaya University. This study used commercial feed of fish with a protein content of 32%. Prebiotics used are derived from sweet potato extract with total dissolved solids (TPT) of 5%, while probiotics with density 10^8 CFU.mL⁻¹ [5].

This research uses a Complete Factorial Randomized Design (RALF), which consists of two factors with six treatments and three replays. Factor A with addition (prebiotics by 1% [7] + probiotic *Bacillus megaterium* of 20 mL.kg⁻¹ of feed [8] at a concentration of 10^8 CFU.mL⁻¹ with a fermentation time of 24, 48, and 72 hours), and factor B (prebiotics by 2% [9] + probiotic *Bacillus megaterium* of 20 mL.kg⁻¹ of feed [10] at a concentration of 10^8 CFU.mL⁻¹ with a fermentation time of 24, 48, and 72 hours).

The analysis data is obtained from research such as biological parameters (abundance of

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bacteria in fermented feed) where it is processed by using fingerprint analysis (ANOVA) in accordance with the Complete Factorial Random Design (RALF) using Microsoft Excel 2013. If there is a real effect, then the next step is the Smallest Real Difference (SRD) test with a confidence level of 95%. Physical parameters (color, aroma, texture, and general condition of feed) and chemical (fermented feed proximate) are analyzed descriptively.

RESULT AND DISCUSSION

Physical Analysis

Physical observation consists of the color, aroma, texture, and general condition of prebiotic and probiotic fish feed against physical qualities based on the different fermentation time lengths. The results of the study are shown in Table 1. It shows the physical condition of feed that has not changed in the physical state for the treatment of factors A and B compared to control. Factors A and B with fermentation time from 24 to 72 hours, on the color, parameters indicate the physical state of brown feed. Feed aroma has a distinctive smell such as the fishy smell of fish, normal texture, not soft and easily destroyed, and for the general condition, the feed shows the normal conditions with feed conditions that do not clump and unmold. Based on the results of the previous study [3], feed containing probiotics with time of fermentation of seven days did not experience physical changes. It was resulting in a normal texture, unsoft and easily destroyed with brown feed color, feed aroma is most likely a fishy smell of fish and general condition feed does not clump, and there are no fungi.

The good quality of the feed, physically, has a distinctive smell, brown color, and no fungi [10]. Feed that undergoes fermentation process for 22 to 34 hours has a brown color [11]. The standard aroma and color of fishmeal that is suitable for use are that it has a distinctive aroma such as fishy smell and the color looks mostly like yellowish-brown to dark brown [12].

Chemical analysis

Based on the fish feed proximate analysis method [13] in Table 2, the change in nutrient content in the feed that is added prebiotics and probiotics during the fermentation process, occur from the hour of 24th to 72nd. Ash content in factor A decreased after fermentation of the hour of 24th to 72nd from 9.67% to 9.45%, while factor B of the hour 24th, ash content by 9.69% increased in 48 hours to 9.72 and decreased in 72 hours by 9.53%. but the results of the analysis of ash levels in a whole, both factors A and B decreased compared to the treatment without the addition of prebiotics and probiotics and did not undergo the fermentation process. The decrease in ash content is caused by the activity of microbial growth due to the fermentation process that occurs in feed. Good ash content as feed is when it has low ash content because it will be easy to digest by the fish [14]. In protein, fats, and carbohydrates increased after fermentation in both factors A and B. Protein content increased from 32.14% (before fermentation), increased at the hour of 24th in factor A by 32.60% and factor B by 33.58%, by the hour of 48th, it is increased in factor A by 32.90% and factor B by 33.95% and increased again until the hour of 72nd in factor A by 33.13% and factor B by 34.14%.

Table 1. Physical analysis of fish feed

Parameters	No Fermentation	Fermentation Time (hours)					
		A1 (28 hours)	A2 (28 hours)	A3 (72 hours)	B1 (48 hours)	B2 (72 hours)	B3 (72 hours)
Color	Brown	Brown	Brown	Brown	Brown	Brown	Brown
Aroma	Feed Aroma	Feed Aroma	Feed Aroma	Feed Aroma	Feed Aroma	Feed Aroma	Feed Aroma
Texture	Normal	Normal	Normal	Normal	Normal	Normal	Normal
General Conditions	Normal	Normal	Normal	Normal	Normal	Normal	Normal

Table 2. Chemical analysis (fermented feed proximate)

Feed (%)	No Fermentation	Fermentation Time of Feed (hours)					
		A1 (24 hours)	A2 (48 hours)	A3 (72 hours)	B1 (24 hours)	B2 (48 hours)	B3 (72 hours)
Ash	9.96	9.67	9.50	9.45	9.69	9.72	9.53
Coarse Protein	32.14	32.60	32.90	33.13	33.58	33.95	34.14
Fat	6.29	7.08	7.09	7.59	7.00	7.06	7.34
Carbohydrates	49.45	50.06	50.12	50.91	50.27	50.73	50.99

Based on these results, it can be concluded that the highest protein content is found in factor B, with a fermentation time of 72 hours of 34.14%. The increase of crude protein in fermented feed as a result of the growth process of microbial cells that develop in feed is seen in Table 3. The possibility of crude proteins to increase is due to the increasing growth of cells of the microbes during the fermentation process [15]. The growth of probiotic bacteria cannot be separated by prebiotics because probiotics desperately need prebiotics to spur their growth [16]. It is suspected that in the fermentation process, there is exogenous enzyme activity by means of macromolecular hydrolysis reactions into simpler molecules such as proteins into amino acids. Microorganisms that can adjust to the surrounding environment are rich in complex molecules by secreting exogenous enzymes by catalyzing macromolecular hydrolysis reactions into simpler molecules such as proteins into amino acids [17].

Fat content increased from 6.29% (before fermentation), it increased at the hour of 24th in factor A by 7.08% and factor B by 7.00%, by the hour of 48th, it increased in factor A by 7.09% and factor B by 7.06%, then it increased for more at the hour of 72nd in factor A by 7.59% and factor B by 7.34%. The increasing levels of fats are suspected due to the microorganisms that are occurred from living cells are able to produce microbial oil or fat. During the fermentation process, the fat content increases due to microorganisms that are able to produce microbial oil, where microorganisms occurred from the living cells are able to produce lipids or fats [18].

Carbohydrate content increased from 49.45% (before fermentation), increased at the hour of 24th in factor A by 50.06% and factor B by 50.27%, at the hour of 48th, it increased in factor A by 50.12% and factor B by 50.73% and increased for more at the hour of 72nd in factor A by 50.91% and factor B by 50.99%. Increased carbohydrate levels are caused by carbohydrate content i.e. oligosaccharides derived from sweet potato extract can affect the growth of *Bacillus megaterium* bacterial activity during the fermentation process. It is suspected that with the addition of oligosaccharides, bacteria will grow to the maximum and the activity of exogenous enzymes that work in the fermentation process is catalyzed macromolecular hydrolysis reactions into simpler

molecules, such as polysaccharides into sugars. Probiotic bacteria can produce exogenous enzymes such as cellulase, amylase, lipase, and protease [19].

Based on the analysis of feed chemically, it can be concluded that fermentation technology conducted with the addition of prebiotics (sweet potato extract) by 2% and probiotics (*Bacillus megaterium*) as much as 10^8 CFU.mL⁻¹ in the feed may increase the content of nutritional level such as proteins, fats, and carbohydrates that can be used as animal feed or fish. Fermentation is a process of breaking down organic compounds into simpler compounds by involving microorganisms. This organic compound consists of carbohydrates, fats, proteins, and other organic matters undergoing a process of chemical changes in an aerobic and anaerobic state through the work of enzymes produced by microbes [20]. Protein levels increase because microbes have the ability to convert complex proteins into simple compounds such as amino acids with the help of protease enzymes. High nutritional levels among others will undergo the fermentation process first by the process of breaking down the food ingredients that contain fats, carbohydrates, and proteins, which are difficult to digest to be easier to digest, and there is a distinctive smell and aroma [21].

Biological Analysis

SRD_{0.05} test results of the abundance of bacteria at the hour of 24th, 48th and 72nd, after the fermentation process of feed showed a noticeable effect on the length of time fermentation of feed containing prebiotics and probiotics. The abundance of bacteria on the main effect of feed fermentation time showed the treatment 3 (72 hours) differed markedly higher than the other treatments. In interactions between B3 treatment factors differed markedly higher if compared to the other treatments.

The duration of fermentation of prebiotic administration at a dose of 1% with probiotics of 20 mL.kg⁻¹ affects 97% (coefficient of determination = 0.97) against the abundance of bacteria. Meanwhile, the administration of prebiotic doses 2% with probiotics of 20 mL.kg⁻¹ affects the abundance of bacteria by 85% (coefficient of determination = 0.85) seen on the chart, where it can be concluded that the longer the the duration of fermentation time taken, the higher the abundance of bacteria obtained.

Table 3. SRD_{0.05} test result of bacterial abundance 10⁻⁴ (CFU.mL⁻¹) in fermented feed

Effect of Single Dose	The Influence of Single Time (SRD _{0.05} = 20.96)			Main Effects of Fermentation Doses (SRD _{0.05} = 14.82)
	1 (24 hours)	2 (48 hours)	3 (72 hours)	
A (1%)	65 ^a	118 ^b	179 ^c	121 ^a
B (2%)	271 ^d	441 ^e	477 ^f	396 ^b
The Main Influence of Fermentation Time (SRD_{0.05} = 12.10)	168 ^a	279 ^b	328 ^c	

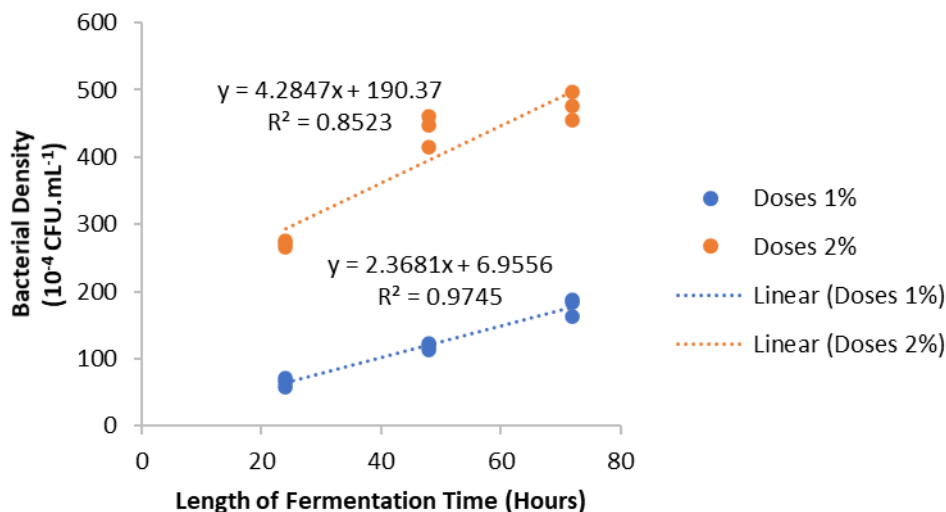


Figure 1. Abundance of fermented feed bacteria

The length of fermentation time is directly related to the growth of microbes that will undergo a phase that changes every time and optimizes the temperature. Extending the fermentation duration time in the fermentation process may be able to provide opportunities for microbes to remodel the components inside the substrate into simpler components to digest. The microbes will undergo a change in growth that binds directly with the increase in the number of microbes as they increase in the number of cells by utilizing the nutrients that have been broken down into simpler sugars forms which can be used as a source of energy [22].

Based on the results of this study, B3 treatment has the highest content of feed ingredients such as protein (43%), fat (7.34%), and carbohydrates (50.99%). According to Indonesian National Standards (SNI) [23], SNI 01-7242-2006 protein content of 25-30% and fat content of at least 5% in tilapia enlargement. Feed ingredients necessary for optimal growth and health of fish such as protein (38-42%), carbohydrates (30-40%), and fat (7-15%) [24].

CONCLUSION

The length of time of fermented fish feed, which is added prebiotics and probiotics, has a noticeable effect on the abundance of bacteria. The length of fermentation time is good for the physical, chemical, and biological state of feed i.e. B3, with a fermentation time of 72 hours that can increase protein and bacteria number to be more abundant.

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The Effectiveness of Weed as Beetle Bank Against Abundance of Soil Arthropods on Corn (*Zea mays. L*)

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Abstract

Conservation of natural enemies on maize can be done by utilizing grass weeds as Beetle banks. Natural enemies of shelter and get food from the weed grasses that are in the land irrigated corn. Natural enemies observed in this research were the predator of ground arthropods in the form of ground beetles, tomcat, and predators arthropods in other lands. The trap used is the Pitfall trap. The percentage of arthropod predators was 69%, and arthropods herbivore was 31%. The abundance of soil arthropods in the treatment of weeds grass is higher than the control treatment It is because of the treatment of weed grass soil arthropods get habitat for a place to stay. Weed grasses provide food sources for the arthropods and get protection from an environment that is less supportive. The use of weed grasses in addition to Beetle banks also serves as a place to breed. The highest abundance of arthropods was situated on the treatment *Eleusine indica* that has a lush lawn and has a characteristic odor favored by arthropods. Arthropods that acts as a predator in the land are the family of Formicidae, Philodromidae, Carabidae, Staphylinidae, Pentatomidae. Some Arthropods from the Pentomidae family have a role as a pest and others as natural predators or predators. Arthropods found in the research was an arthropod that had a role as natural enemies or predator. The Pentomidae family that has a position as a predator is *Picromerus Bidens*. Ground beetles were found in the *C. fossor* and *Pheropsophus* sp. Ground beetles are dominant picking weeds *Setaria* sp. and *Eleusine indica* as habitat and place in search of food and life's survival.

Keywords: Beetle bank, soil arthropods, weeds.

INTRODUCTION

Corn production has been increased, although the decline is also occurred caused by various factors, one of which is the presence of pests and diseases of plants. Pest and disease control made much use of chemical pesticides. It needs to be reduced so that the pest population is not increasing. Pest and disease control can be done by using resistant varieties, planting time, and using biological control so it will not cause damage to the ecosystem [1]. Biological control can be defined as a business in pest control. It was done with biological action, for example, with the utilization of natural enemies of both the predators and parasitoids that are used to suppress populations of target pests. Biological control is done by utilizing the components of the biotic techniques, such as the conservation of natural enemies of specific pests to be controlled [2].

Conservation of natural enemies was conducted to increase the population of natural enemies and help in suppressing the population of the pest so that it can fix the diversity of the ecosystem. This activity is done to provide a

supportive environment for the survival of naturally occurring, mainly in providing the availability of feed. This activity can also be done by setting the cropping system and cropping pattern, do the intercropping between corn plants with other plants, and the utilization of the plant is flowering or refugia as towing natural enemies [3]. Beetle banks are one of the techniques of conservation control biologically, which aims to increase the population of natural enemies in controlling pests in plants [4]. Beetle banks can provide habitat for natural enemies so that the population of predators increased and pest populations low. Beetle banks are a plant grass planting performed with elongated in cropland and aims as the habitat of the predator, for example, ground beetles [5].

The use of grass weeds in the outskirts of the plant can be used as a habitat for predators to take place to live up next to the planting [6]. Weed grass could be a determinant for the presence of natural enemies of both predators and parasitoids because the plant acts as a protector and host for natural enemies of both predators and parasitoids [7]. Weed grasses can also act as a source of feed supplement and as a place to lay their eggs for the predators to breed.

The use of grass plants as a habitat for predatory ground beetles can be used as a strategy in the conservation of land and the

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ecosystem [8]. The type of weed can affect its effectiveness in increasing the diversity of the population of arthropods on the ground. The weed used are *Setaria* sp., *Eleusine indica*, *Cyperus rotundus*, *Echinochloa crus-galli*, and *Leptochloa chinensis*. The use of various types of weeds can affect the populations of natural enemies that come in and affect the number of eggs laid in the corn crop [9].

Weed grasses that have been studied to be used as beetle banks were *Setaria* sp., *Trifolium pretense*, and *Taraxacum officinale* [8]. Weeds can bring ground beetles to cropland. A species of ground beetle found in the previous research were *Pseudophonus rufipes* and *Harpalus differendus* [8]. This research was aimed to determine the effectiveness of weed *Setaria* sp., *Eleusine indica*, *Cyperus rotundus*, *Echinochloa crus-galli*, and *Leptochloa chinensis* in increasing the abundance of soil arthropods in maize. The

effectiveness or success can be seen from the type of weed used and rely on extensive Beetle banks that are used for the habitat of the ground beetles.

MATERIAL AND METHOD

Trial Plot

The preparation was done is to determine the treatment land of corn plants. We used a microscope, a camera, a bottle of mineral water, rope, stake, stationery, tweezers, and other tools that support the research. The necessary seeds included corn seeds, seeds of weeds *Setaria* sp., *Eleusine indica*, *Cyperus rotundus*, *Echinochloa crusgalli*, and *Leptochloa chinensis*. We also used alcohol, detergent, plastic bag, organic fertilizer, soil. The research design used Randomized block Design experimental plots, each measuring 1.6 m x 2 m with a distance treatment of 2 m (Fig. 1).

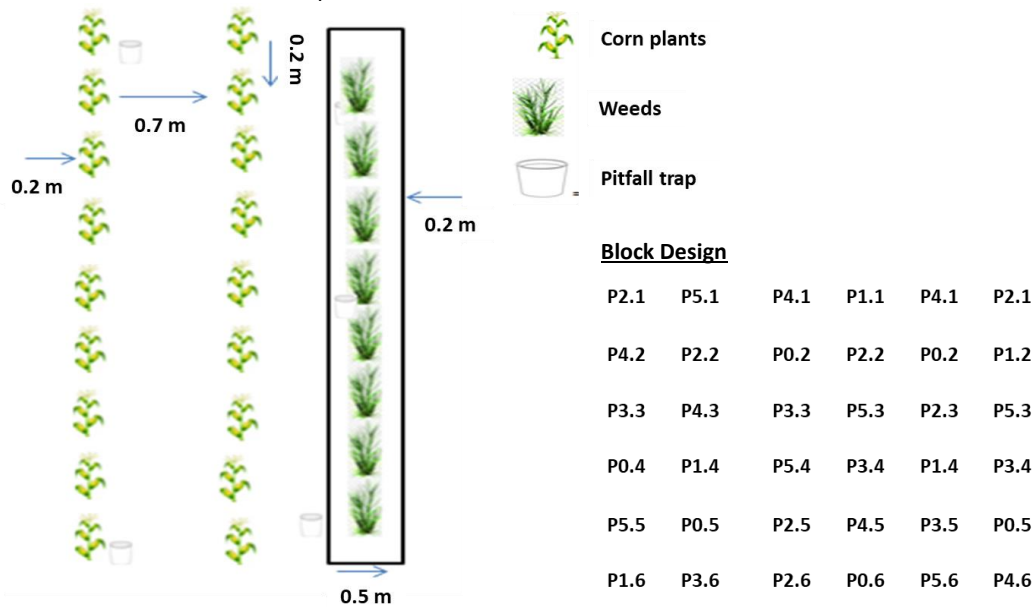


Figure 1. Trial plot plan



Figure 2. Pitfall traps

Data Collection

Observed variables measured are the population of soil arthropods, the diversity of soil arthropods, the abundance of arthropods on the ground. The observations made that use trap hole trap or pitfall trap (Fig. 2). Pitfall traps or hole traps were used to catch predator insects that are active at the surface of the soil. Pitfall trap was made of plastic cups volume of 250 ml, which is filled with detergents that are laid on the surface of the soil in the rice fields. Traps are installed in every corner of the map and mounted each morning at 07.00 PM. Insects captured were collected and put in a plastic bag or bottle of aqua used then taken to the laboratory to be identified insects. The collection of insects was done every seven days, starting from 14 days after planting until harvest.

Insect Identification

Insect identification was performed in the Laboratory of Agrotechnology, Faculty of Agriculture, University of Jember. Identification was done by book guide through the site

Bugguide.net [10] and see the guidebook Borror *et al.* [11].

RESULT AND DISCUSSION

Observation of the arrival of arthropods on weed grasses in corn planting was held at the age of 14 up to 77 days after planting. Soil arthropods found comes from five orders, i.e. Hymenoptera, Araneae, Coleoptera, Hemiptera, and Orthoptera. These orders were into six families, namely Formicidae, Philodromidae, Carabidae, Staphylinidae, Pentatomidae (Fig. 3).

The arrival of ground arthropods at the plant will be increased when the habitat and food of the arthropod can be met so that the population of arthropods increased. Habitat or place of residence for ground arthropods can be done by providing the plant herbage called beetle banks. The highest percentage of order found was Araneae by 38%, then followed by Coleoptera by 31% and Hymenoptera by 25%. The lowest percentage of orders found was Orthoptera and Hemiptera of each 3% (Fig. 4).

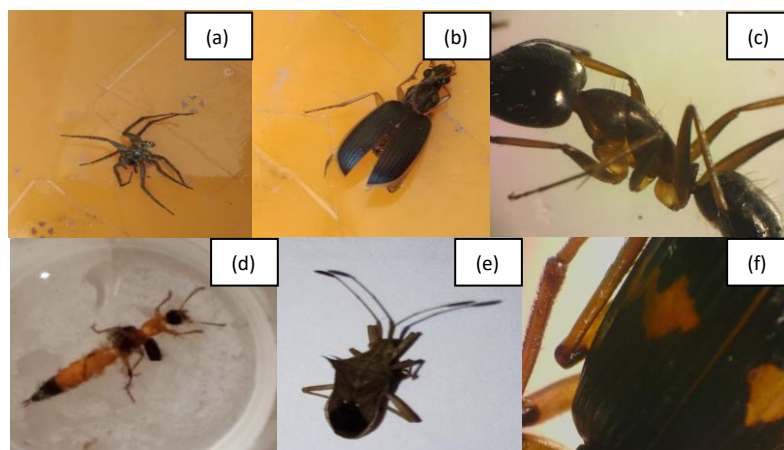


Figure 3. Soil arthropods: (a) Famili Philodromidae, (b) Famili Carabidae, (c) Famili Formicidae, (d) Famili Staphylinidae, (e) Famili Pentatomidae, (f) Famili Carabidae

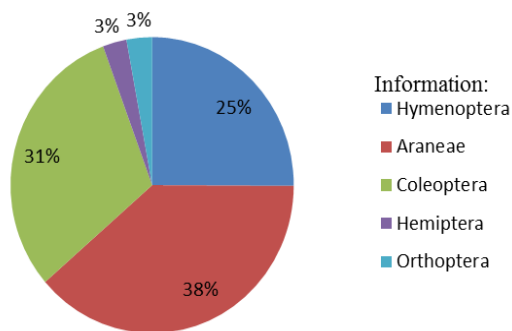


Figure 4. The total percentage of soil arthropods in corn cultivation based on insect order

The percentage of the Hemiptera ranks was low due to habitat for arthropods did not support the survival of arthropods. Arthropods from the Hemiptera Order do not like dry and hot ground so that when grown in cornfields, the habitat does not support its survival [12]. The attracted Coleoptera order is quite high. It was because the environmental conditions are sufficient, which include moisture, temperature, and precipitation. The population of soil arthropods will be increased when precipitation is high, therefore when the dry season, the population decline [13].

The percentage of attracted arthropod predators was mostly found in wild plants than

plant corn (Fig. 5). It is because in the wild plant, food is available, and arthropods get protection from the grass. Thus, they are like living in wild plants more. At the corn crop, there is a competition of food with pests.

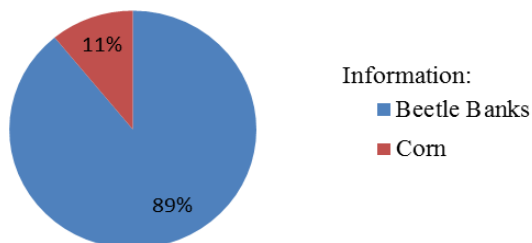


Figure 5. The percentage of attracted predators arthropod on weed and corn crop.

Methods that can keep the *Eleusine indica* grass growth well is by continuously taking care of it—watering it so that it will not wither and seeding from that grass till it can grow well and prolific. Therefore, *Elusiane indica* can be constantly used with years of the life cycle so that breeding can be conducted on them.

The predator population was very dependent on the place of residence and the source of food as well as shelter. The provision of shelter and

food sources is needed with the use of wild plants as microhabitats so it can increase the population of predators. The weed was derived from weed grass. Family Poaceae is the family of a grasses group that can be used as an alternative habitat provider of food to increase the population of predators. The abundance of arthropods in each observation is different. It was caused by the availability of food and the habitat of the weed. The highest abundance of arthropods was located on the weeds age 30-45 days after planting because at that age, the availability of food of weed is still abundant, and weed growth is still better. Therefore, the predator has enough food to survive [14].

Predators found in the beetle bank were *Philodromus* sp, *Camponotus* sp, *Paederus fuscipes*, *Astenus* sp, *Clivina fossor*, and *Pheropsophus* sp. Ground beetles found in the corn crop were family Carabidae, i.e. *C. fossor* and *Pheropsophus* sp. (Table 2). The population of ground beetles found is still relatively low. It is because the habitat and availability of food are not sufficient for the survival of ground beetles [15].

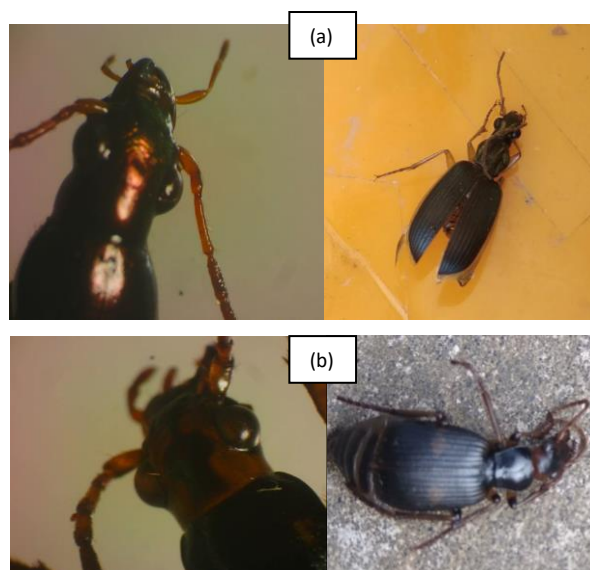


Figure 6. Ground beetles found; (a) *Clivina fossor*, (b) *Pheropsophus* sp.

Table 2. Population of Ground Beetle in corn crop

No	Ground Beetle	Function	Population (Indiv.)
1	<i>Clivina fossor</i>	Predators	34
2	<i>Pheropsophus</i> sp.	Predators	95

There are biotic and abiotic factors that cause the population of ground beetles to decrease. The survival of ground beetles is determined by the habitat of the ground beetles, namely the availability of wild plants preferred by ground beetles [16]. The population of ground beetles, along with the creation of suitable habitat, also depends heavily on the existence of temperature in the environment around. Ground beetles are active at the beginning of the summer will be predators important in the active predation of crop pests [17]. Ground beetles *C. fossor* and *Pheropsophus* sp. found in the cropland serve as predators in maize. The existence of ground beetles *C. fossor* and *Pheropsophus* sp. can help to reduce or suppress the population of pests' family Noctuidae, which damage the corn crop. *Clivina fossor* and *Pheropsophus* sp. can be agents of biocontrol or biological control that is able to suppress the pest population [18]. *Clivina fossor* and *Pheropsophus* sp are soil arthropods from the Carabidae family, which have a role as predators. *Pheropsophus* sp is a bug that attacks stem borers in corn plants [19].

Index of arthropod diversity (H') in all treatments has intermediate results. In this index (table 2), the diversity of all treatments was with a value of $1 < H < 3$. The lowest value of diversity found in the treatment of P5 *Leptochloa chinensis* amounted to 1.57, and the highest value of diversity index was located on the P2 treatment *Eleusine indica* that is equal to 1.70. The low index value diversity means there are no species that dominate and the availability of food for arthropods. The layout of a corn plant that is adjacent to the beetle bank will also greatly affect the value of the diversity. The value of the index of diversity will be low when the location of the adjacent. The value of the index of diversity will be low when the location is adjacent. It is caused by the absence of a significant difference for arthropods to make the shift in their existence so that the diversity of the medium [20].

Diversity is relatively low because the research ground is only composed of a few species. Meanwhile, if the ground is lapped by many species, then their diversity will increase [21]. The relative abundances of soil arthropods cropland were low (0.1). We found that the highest diversity of ground Arthropods lies in the family of Philodromidae, namely spiders as much as 212 individuals. The second-highest abundance of arthropods lies in the family of Formicidae as many as 138 individuals. The

abundance of arthropods is relatively low because the value of biodiversity is low, and because of the availability of food in the land insufficient for arthropods so that their abundance in the land is low [22]. The low value of abundance is also caused by the installation of the trap that is exposed to rainwater so that the soil is wet, which resulted in the position of the bottle is higher than the ground so that the arthropods are more difficult to get into the trap. The use of weed grasses was not only attracted one specific species of arthropod but general species so that the value of each species abundance was low. Weeds that act as *Beetle banks* attract more than one species (general arthropod species).

Table 3. Diversity index and relative abundance of Arthropods

Treatment	H' (Diversity Index)	Relative Abundance
Control	1.69	0.1
<i>Setaria</i> Sp.	1.67	0.1
<i>E. indica</i>	1.70	0.1
<i>C. rotundus</i>	1.59	0.1
<i>E. crusgalli</i>	1.64	0.1
<i>L. chinensis</i>	1.57	0.1

CONCLUSION

The use of weed grasses could provide a habitat for ground beetles to survive and provide food for beetles the ground to breed. Treatment that is effective in providing habitat for ground beetles is *Eleusine indica* and *Setaria* sp., followed by control treatment, *Cyperus rotundus*, *Echinochloa crusgalli*, and *Leptochloa chinensis*. Ground beetles that were found included in the family of Carabids, namely *C. fossor* and *Pheropsophus* sp. Weeds can be used as Beetle banks to provide habitat for ground beetles in the irrigated corn land. The abundance of the highest population on *Eleusine indica* treatment was 120 individuals. The lowest value of diversity was in the *Leptochloa chinensis* treatment for 1.57, and the highest value of the diversity index was in *Eleusine indica* treatment for 1.70. The relative abundance value of soil arthropods in cropland was low, i.e. 0.1.

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Antimicrobial Activity of Combination Bacteriocin and Asam Sunti Extract (*Averrhoa bilimbi* L. fermented) Against Multidrug Resistant *Escherichia coli* in Lettuces (*Lactuca sativa*)

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Abstract

The ready-to-eat vegetables are often associated with the presence of multidrug-resistant (MDR) bacteria. This study aimed to evaluate the potency of bacteriocin, Asam Sunti extract, and their combination against MDR *E. coli* in lettuce. Their antimicrobial activity was assessed using the disk diffusion method and bacterial enumeration after direct application in pre-inoculated lettuce with MDR *E. coli*. The bacteriocin was produced by *Lactobacillus plantarum* BP102 at optimum production time or during the stationary phase at 18 h. These bacteriocins were able to inhibit five MDR *E. coli* isolates, while Asam Sunti extract and the combination of bacteriocin and Asam Sunti extract were only able to inhibit three MDR *E. coli* (LL1.2, LL3.11, and LL3.12) and (LL1.2, LL1.3, and LL3.11), respectively. In direct application to pre-inoculated fresh lettuce, higher inhibition of MDR *E. coli* was observed after applying the combination of bacteriocin and Asam Sunti extract with a ratio of 1:1 and 1:2, compared to bacteriocin alone. However, the inhibitory activity of this combination treatment was not significantly different ($p>0.05$) with the Asam Sunti extract alone. The highest rate of decrease in total bacteria in lettuces was 97% occurred in isolate LL1.2 with bacteriocin treatment alone, and isolate LL3.11 with combination treatment of bacteriocin and Asam Sunti extract (1:2). While on MCA media, the best reduction rate of 94% occurred in isolate LL1.2 with treatment using bacteriocin only, Asam Sunti extract only, and their combination (1:2). The inhibition of MDR *E. coli* in fresh lettuces by bacteriocin, Asam Sunti extract, and their combination was strain-dependent which was indicated by various inhibition results in all treatments.

Key words: Asam Sunti extract, Bacteriocin, multidrug resistant.

INTRODUCTION

The emergence of multidrug-resistant bacteria (MDR), where microorganisms are resistant to more than one antibiotic, has become a problem that needs attention. The MDR bacteria are usually found in health-related facilities. Surprisingly, the emergence of MDR bacteria is also reported in foods [1,2,3]. The MDR bacteria found in fresh or raw foods such as vegetables might be due to the natural contamination from irrigation water, organic fertilizers, and soil during cultivation [4].

Fresh vegetables such as lettuce are commonly consumed by Indonesian people as *lalapan* and used as raw materials in the salad. Fresh vegetables that are washed only with water are not properly able to eliminate pathogenic bacteria that contaminate the foods, such as *Escherichia coli* [5]. Ready-to-eat vegetables (RTE) such as lettuce, basil, long beans, and cabbage sold in the Malang market are reported to contain MDR bacteria. Lettuce contains MDR *E. coli* bacteria that are resistant to

kanamycin, tetracycline, and streptomycin [6]. In 2018, in the United States and Canada, health problems associated with the consumption of fresh romaine lettuces as reported by The Centers for Disease Control (CDC) that out of 58 people who were sick, five were hospitalized, and one person died. The fresh lettuces were contaminated with *E. coli* O157:H7. The *E. coli* O157:H7 strain was found in the sediment of the agricultural water reservoir on the farm [7]. MDR bacteria such as gram-negative bacteria that produce ESBL (Extended Spectrum Beta-Lactamase) enzymes, such as *E. coli* and *Klebsiella pneumoniae*, can destroy many clinically important antibiotics. Bacteria expressing ESBL are difficult to control using more than two or three antibiotics [8].

Bacteriocins are a potential candidate to replace antibiotics as antimicrobial agents against MDR bacteria [9]. However, bacteriocins are less effective in controlling the growth of Gram-negative bacteria such as MDR *E. coli*. The outer membrane of these bacteria acts as a barrier to cell permeability towards antimicrobial substances reaching the cytoplasmic membrane. In addition, bacteriocin activity is also influenced by the presence of the proteolytic enzyme produced by those bacteria [10]. Therefore,

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bacteriocin alone does not guarantee its efficacy in inhibiting Gram-negative bacteria such as MDR *E. coli*. The combination of bacteriocins with other antimicrobial compounds and physical treatments that can work synergistically, providing better activity [11]. The combination of bacteriocin (nisin) and essential oil of *Ocimum basilicum*, *Salvia officinalis*, and *Trachyspermum ammi* showed a synergistic interaction in inhibiting the bacteria *E. coli* O:157 [12].

Asam Sunti is a fermented starfruit (*Averrhoa bilimbi* L.) used as a spice in food by the people in Aceh Province. Asam Sunti contains organic acids such as oxalic acid, malic acid, lactic acid, citric acid and ascorbic acid. In addition, it also contains phytochemical compounds with antimicrobial activity such as alkaloids, tannins, flavonoids, phenols, and saponin can be used as antibacterial agents [22]. The combination of bacteriocin with natural compounds such as Asam Sunti extract is expected to increase its effectiveness in inhibiting MDR bacteria. Thus, the purpose of this study was to evaluate the potential of the combination of bacteriocin and Asam Sunti extract in inhibiting MDR *E. coli* bacteria in lettuce.

MATERIAL AND METHOD

Bacterial Cultures

Lactobacillus plantarum BP102 was a bacteriocin producer isolated from garlic bulbs (*Allium sativum*) [13]. Indicator pathogen bacteria used were MDR *E. coli* strain LL1.2, LL1.3, LL3.11, LL3.12, and LL3.13, which were isolated from RTE vegetables from Malang markets [6].

The Optimum Production of Bacteriocins

The liquid culture of *L. plantarum* BP102 (10 mL) was inoculated in 90 mL of MRS broth and incubated at 37°C for 36 h. The bacterial culture was taken as much as 5 mL every hour for six hours and then four hours up to 38 hours, then the optical density (OD) was determined using a spectrophotometer with a wavelength of 600 nm. The bacterial cultures were centrifuged at 10.000 rpm for 20 minutes at 4°C. The supernatant was taken, and the pH was adjusted to pH 6.5. The neutralized supernatant was filtered using a sterile membrane filter (0.22 µm) to obtain a cell-free supernatant (CFS). The inhibitory test was carried out using agar disk-diffusion to determine the bacteriocin activity (Equation 1). After that, the CFS was freeze-dried to be used for further tests [14].

$$\text{Bacteriocin activity (mm}^2\cdot\text{mL}^{-1}) = \frac{Lz-Ls}{V} \dots\dots\dots(1)$$

Description:

Lz = Diameter of clear zone (mm²)

Ls = Diameter of blank disk (mm²)

V = Volume of sample (mL)

Extraction of Asam Sunti

The extraction of Asam Sunti was carried out by the maceration method using ethanol as a solvent [34]. Then, the Asam Sunti extract in the form of paste with a pH of 1.3 was adjusted to pH 2.5, which was then freeze-dried. The salinity of Asam Sunti extracts was also measured using a refractometer.

Phytochemical Analysis of Asam Sunti Extract

The analysis of Asam Sunti extract was carried out by qualitative test according to the Harborne (1987) method. It detected qualitatively the presence of metabolite compounds, such as tannins, phenols, saponins, steroids, alkaloids, and flavonoids.

The Combination of Bacteriocin and Asam Sunti Extract *in Vitro*

One loopful of MDR *E. coli* isolates as indicator bacteria was taken and inoculated in 25 mL Nutrient broth incubated at 37°C for 24 h. The indicator bacteria (0.1 mL) with a cell density of 10⁶ cells.mL⁻¹ were inoculated into Nutrient agar using the spread plate technique. In this experiment, three treatments were used, namely 1% bacteriocin, 1% Asam Sunti extract, and a combination of bacteriocin and asam sunti extract (v/v) with a ratio (%) of 1:1, 1:2, and 2:1. Method for preparing a combination immersion solution of bacteriocin and Asam Sunti extract (1%) for immersion were prepared by dissolving 1 g of bacteriocin or Asam Sunti extract in 100 mL of sterile distilled water. The 50 µL of each sample was used for antimicrobial activity test using the agar disk diffusion method [15]. The antimicrobial activity was calculated using Equation 1.

Application of Bacteriocin and Asam Sunti Extract on Lettuces

Fresh lettuces (2 g) were artificially contaminated by immersing in 10 mL NB medium containing 10⁶ cells.mL⁻¹ of MDR *E. coli* at room temperature for 5 mins. The strain of MDR *E. coli* used was the sensitive strain according to the result of *in vitro* tests. Then, the lettuce samples were then placed on sterile filter paper. Each contaminated lettuce was then immersed for 5 mins in 5 mL of 1% bacteriocin, 1% Asam Sunti extract, and a combination of both. While fresh

lettuces (2 g) immersed in 10 mL of sterile distilled water were used as the control. Lettuce sample was rinsed with 5 mL of sterile salt water (0.85% NaCl) before it was diluted 10-fold and inoculated into Nutrient agar (to detect total of bacteria) and MacConkey agar (selective medium used for the detection of *E. coli* that can be seen from the characteristic morphology) using the pour plate method with three replications. Petri dishes were incubated at 37°C for 24 h, and colony counts were conducted using total plate count [14]. The percentage of reduction of the total number of bacteria growing on the media was calculated according to Equation 2.

$$\text{Cell number reduction (\%)} = \frac{N_0 - N_t}{N_0} \times 100\% \dots\dots\dots(2)$$

Description:

N_0 = Number of bacterial colonies in the control.

N_t = Number of bacterial colonies in the treatment.

Data Analysis

The data obtained from the *in vitro* test and application of fresh lettuce were analyzed using a one-way analysis of variance (ANOVA) with $p < 0.05$. The results that are significantly different were further tested using Tukey's test. Data analysis was performed using SPSS 21.0 for Windows.

RESULT AND DISCUSSION

Bacteriocin Activity and its Growth Curve

The growth curve of *L. plantarum* BP102 (Fig. 1) shows the adaptation/lag phase that occurred at the first hour of incubation. The exponential phase occurred at 2 h to 10 h of incubation time and was followed by the stationary phase at 10 h to 38 h. The bacteriocin activity of *L. plantarum* BP102 was detected during the exponential and stationary phase, but it reached the optimum activity at 18 h during the stationary phase indicated by the highest inhibition activity against

E. coli LL1.2 and LL1.3. Similar results were reported that the optimum production of bacteriocin of *L. plantarum* was produced after 14 h of incubation [16]. Other studies have shown that the optimum production of bacteriocins was at 19 to 30 h, depending on the high biomass growth medium [17].

The incubation time (Fig. 1) showed the different inhibitory activities. However, the inhibitory activity shown was not much different. The highest inhibitory activity against the two test bacteria was found at 18 h. In the test, the highest inhibition of bacteriocin against *E. coli* LL1.2 was 1.22 mm².mL⁻¹ and for *E. coli* LL1.3 was 1.16 mm².mL⁻¹. The more incubation time, the more bacteriocin activity increased and reached its optimum in the stationary phase.

A study reported that *L. plantarum* ATCC 8014 can produce metabolite compounds that inhibit Gram-positive (*Staphylococcus aureus* and *Listeria monocytogenes*) and Gram-negative (*E. coli* and *Salmonella typhimurium*) bacteria. Tests were carried out at 15, 18, 21, and 24 h, all of which indicated an inhibition zone. The highest zone of inhibition was found at 24 h in three bacteria (*S. typhimurium*, *S. aureus*, and *L. monocytogenes*), but in *E. coli*, the highest inhibition zone was at 18 h [18].

Another study reported that the optimum bacteriocin production in de Man Rogosa and Sharpe Broth (MRSB) at 12 h during the final exponential growth phase and maximum at 32 h during the stationary phase. [19]. Another study reported the optimum production of bacteriocins by LAB at an incubation time of 24 h and a maximum of 48 h. Therefore, the bacteriocin activity depends on the type of bacteria and media conditions [20].

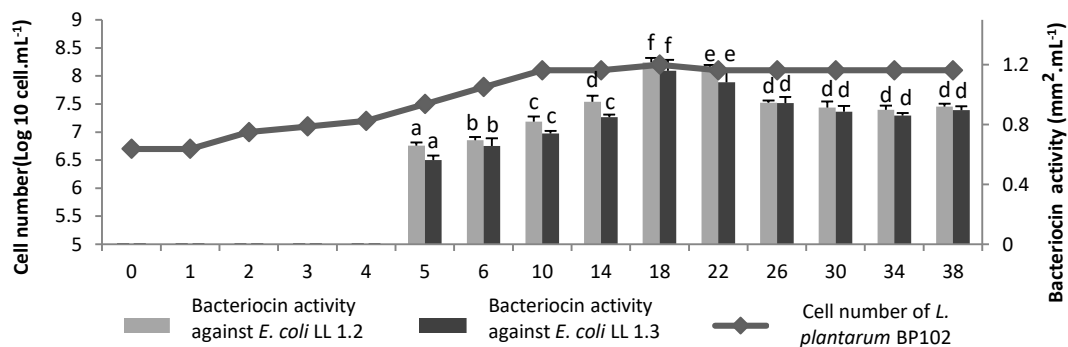


Figure 1. Growth curve and bacteriocin activity of *L. plantarum* BP102 on MDR *E. coli* LL1.2 and LL1.3. Data were expressed as mean ± standard deviation of the three replications. Different notations showed different among treatments ($p < 0.05$).

Phytochemical Analysis of Asam Sunti Extract

Asam Sunti is used by the Indonesian people, especially the people of Aceh Province, as a flavor enhancer. As fruits in general, Asam Sunti contains organic acids. However, the organic acids in Asam Sunti can decrease due to the processing and storage process, except for lactic acid, which tends to increase. The increase of lactic acid indicated the activity of lactic acid bacteria in Asam Sunti [21]. In this study, the phytochemical compounds contained in Asam Sunti extract (Table 1) were saponin and triterpenoid. Only these compounds were successfully identified, possibly because of the salt content in Asam Sunti. The salt content interfered with the phytochemical analysis, so it is necessary to wash it first before the phytochemical content was analyzed. Saponins are compounds that have a strong surface tension that acts as an antimicrobial by disrupting the stability of the bacterial cell membrane, which causes cell lysis. It is because saponins are semipolar compounds that can dissolve in lipids and water so that these compounds will be concentrated in microbial cell membranes [22]. The saponin content in starfruit extract was higher than that of the leaves and petioles. Saponin compounds found in starfruit (*Averrhoa bilimbi* L.) were triterpene saponins [23].

Table 1. The phytochemical compounds in Asam Sunti extract based on qualitative test

No.	Phytochemical compounds	Result
1.	Flavonoids	-
2.	Alkaloids	-
3.	Tannins	-
	Terpenoids	-
4.	Steroids	-
	Triterpenoids	+
5.	Phenol	-
6.	Saponins	+

Description: (+) = Identified, (-) = Not Identified

Combination of Bacteriocin and Asam Sunti Extract *in Vitro*

Based on the qualitative test results of the combination of bacteriocin and Asam Sunti extract on MDR *E. coli* bacteria, the bacteriocin alone showed inhibitory activity against all isolates of MDR *E. coli* (Fig. 2). The highest inhibitory activity was found against strain LL1.2, by 1.14 ± 0.19 mm ($p < 0.05$) and LL3.12 by 1.91 ± 0.04 mm ($p < 0.05$). Meanwhile, the Asam Sunti extract alone showed inhibitory activity

against three isolates, namely LL1.2, LL3.11, and LL3.12, with the highest inhibitory activity against strain LL3.11 by 0.83 ± 0.06 mm ($p < 0.05$). The combination of bacteriocin and Asam Sunti extract with a ratio of 1:1 (B1+AS1) showed inhibitory activity against three MDR isolates, namely LL1.2, LL1.3, and LL3.11. Whereas the combination ratio of 1:2 (B1+AS2) and 2:1 (B2+AS1) also showed activity against four strains of MDR *E. coli*, namely LL1.2, LL1.3, LL 3.11, and LL3.12. The MDR *E. coli* can be inhibited by bacteriocins and Asam Sunti extract and the combination of both against two strains, namely LL1.2 and LL3.11. The combination of 1:2 (B1+AS2) and 2:1 (B2+AS1) against strain LL3.11 showed better activity than bacteriocin, Asam Sunti extracts alone, and the combination 1:1 (B1+AS1). The inhibitory activity of the combination of bacteriocin and Asam Sunti extract was not consistent. It can be assumed that the defense pattern of each strain of MDR *E. coli* was different (strain-dependent).

The *in vitro* test results on MDR *E. coli* LL3.13 showed that no inhibitory activity by Asam Sunti extract and the combination with bacteriocin, but it could only be inhibited by bacteriocin alone. The ability of the rate of adaptation by MDR *E. coli* LL3.13 may result in decreased sensitivity to the antimicrobial compound used. In Gram-negative bacteria, the inhibitory mechanism is more complex because bacteria have a more complex cell wall structure and thus require a higher concentration. The combination of nisin and *Curcuma zanthorrhiza* essential oil, *Curcuma zedoaria* with a concentration of 4%, showed a bactericidal effect towards *E. coli* FNCC 0091 [24]. Several studies have shown that the antimicrobial activity of nisin (bacteriocin) can often be influenced by several factors, including pH, temperature, composition, structure, and natural microbiota in food [25]. In addition, the decrease of bacteriocin activity may be due to proteases released from cells, protein aggregation, adsorption to the cell surface, or feedback regulation [26].

Bacteriocins showed significant inhibition activity against Gram-negative bacteria such as MDR *E. coli shiga toxin-producing* (STEC), which is the most detected pathogen in the food industry [27]. To be able to control spoilage and contamination caused by pathogenic bacteria, many researchers use bacteriocin as part of the hurdle technology [28].

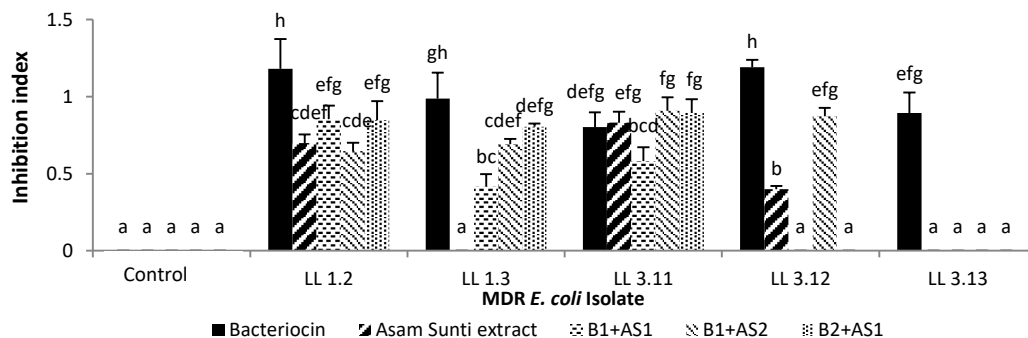


Figure 2. The ability of the combination of bacteriocin and Asam Sunti extract to inhibit MDR *E. coli* bacteria. B1 + AS1: Bacteriocin 1% and Asam Sunti extract 1%, B1 + AS2: Bacteriocin 1% and Asam Sunti extract 2%, and B2 + AS1: Bacteriocin 2% and Asam Sunti extract 1%. Data were expressed as mean \pm standard deviation with three replicates. The bars followed by different letters were significantly different ($p < 0.05$).

Meanwhile, the inhibition activity of Asam Sunti extract can occur by the reaction of metabolite compounds such as saponin, which has antimicrobial activity [29]. Natural antimicrobials, including plant extracts, enzymes, bacteriocins, essential oils (EO), bacteriophages, and fermentable substances, have been shown to have the potential to control pathogenic bacteria in food [30].

Application of Bacteriocin and Asam Sunti Extract on Lettuces

Based on the results of the *in vitro* test, four isolates of MDR *E. coli* bacteria were selected, which had an inhibition zone in each combination treatment (strain LL1.2, LL1.3, LL3.11 and LL3.12) to be used in the application assay. Based on the application study, strain LL1.2 was inhibited by the bacteriocin alone, with the highest reduction percentage by 97% (Fig. 3). Strain LL1.2 was inhibited by Asam Sunti extract alone, with a reduced level of 62%. By using combination of bacteriocin and Asam Sunti extract, the reduction percentage of the number of bacterial cells was 92% in the 1:1 (B1+AS1) treatment and 95% in the 1:2 (B1+AS2) combination. For the ratio 2:1 (B2+AS1) combination, the decrease was only 58% in nutrient agar (total of heterotrophic bacteria).

The highest reduction in the number of LL1.2 bacteria was found in the bacteriocin treatment and Asam Sunti extract, without treatment around 94%, while the highest reduction combination was the B1+AS2 treatment by 92% and B1+AS1 by 85%. The reduction in the number of bacteria isolates LL1.2 on MCA was found in the B2+AS1 combination by 1%, with the number of bacterial colonies of 1.5×10^4 CFU.mL⁻¹, which was the lowest reduction. In the previous *in vitro*

test, the treatment of B2+AS1 against strain LL1.2 showed an inhibition zone. However, when this was treated in lettuces, strain LL1.2 was not inhibited. It might be due to other bacteria originated from lettuce itself (endophytic bacteria).

MDR *E. coli* LL1.3 treated with bacteriocin alone has a reduction percentage of 78%, for Asam Sunti extract alone of 91%, even though the LL1.3 *in vitro* test could not be inhibited by Asam Sunti extract. Whereas the combination of both antimicrobial agents showed the highest reduction in the number of bacteria in the combination of B1+AS2 (93%) and B2+AS1 (97%), while the combination of B1+AS1 the decrease was about 78%, the same as in the washing treatment with bacteriocin alone. The highest decrease in the number of MDR bacteria *E. coli* LL1.3 on MacConkey media was found in asam sunti extract alone by 91%, and the lowest decrease was treated with bacteriocin alone by 87%, the combination B1+AS1 by 89%. The combination of B1+AS2 was 88%, and B2+AS1 was 89%. The reduction in the number of bacterial cells on MacConkey media was not significantly different ($p > 0.05$), especially the decrease in each combination.

Fresh lettuce immersion only in sterile distilled water (control) had a bacterial cell density of 2.5×10^4 CFU.mL⁻¹. MDR *E. coli* LL3.11, which was given bacteriocin alone, decreased the percentage by 10% with the number of cells 2.4×10^4 CFU.mL⁻¹ on nutrient agar media. The effectiveness of bacteriocins in food systems is often low due to several factors such as adsorption to food components, enzymatic degradation, poor solubility, or uneven distribution in the food matrix [31].

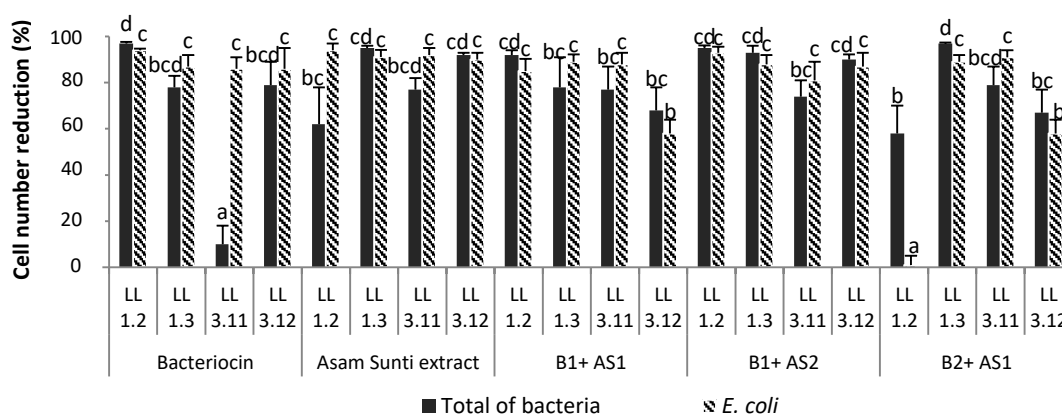


Figure 3. The percentage decrease in the total of bacteria and *E. coli*. Data are expressed as mean \pm standard deviation. The different notations on the histogram indicate a significant difference between treatments ($p < 0.05$).

The decrease in MDR *E. coli* LL3.11 on Nutrient agar media by immersion treatment with Asam Sunti extract alone was 77%, while bacteriocin alone was 10% and the combination of 1:1 (B1+AS1), 1:2 (B1+AS2), 2:1 (B2+AS1) was 77%, 74%, and 79%, respectively. It can be concluded that the reduction in the number of heterotrophic bacteria was not significantly different ($p > 0.05$) in each combination treatment. MDR *E. coli* LL3.11 on MacConkey agar treated with bacteriocin alone decreased by 86%, Asam Sunti extract alone by 92%, and the combination of 1:1 (B1+AS1), 1:2 (B1+AS2), 2:1 (B2+AS1) was 88%, 81%, and 91%, respectively.

The highest reduction in the total number of *E. coli* LL3.12 on Nutrient agar showed in the treatment of Asam Sunti extract alone (without combination) by 92%, bacteriocin alone (without combination) by 79%, and the highest percentage reduction in the combination of bacteriocin and Asam Sunti extract was found in B1+AS2 by 90%, while the lowest percentage in the combination of 2:1 (B2+AS1) by 67%. In MacConkey media, strain LL3.12 had the highest reduction in the number of cells in the treatment of Asam Sunti extract alone by 90%, and the lowest percentage was found in the combination of B1+AS1 and B2+AS1 by 58%. Therefore, it was assumed that there was an effect of the higher concentration of Asam Sunti extract in inhibiting the growth of *E. coli* LL3.12.

The soaking treatment with Asam Sunti extract has the potential to reduce all MDR *E. coli* in lettuce, while the best combination ratio was 1:1 (B1+AS1) and 1:2 (B1+AS2). The inhibition of MDR bacteria by immersing bacteriocin alone experienced the highest decrease against strain LL1.2 both on NA and MCA media. Whereas in

the treatment of Asam Sunti extracts alone, the highest decrease was found towards strains LL1.3 and LL3.12 (NA media) and strain LL1.2 (MCA media), but the reduction value was not significantly different ($p > 0.05$). The combination of bacteriocin and Asam Sunti extract in all comparisons was able to inhibit strain LL1.3 with a relatively high percentage value. The reduction in the number of bacterial cells in both media was considered the best if the percentage was above 80%.

The reduced effectiveness of bacteriocins in inhibiting pathogenic bacteria can be attributed to the development of resistance, their interaction, inactivation, or even binding to the bacterial growth medium [32]. The phytochemical compound, especially saponin, was expected to increase the effectiveness of bacteriocin activity in inhibiting MDR bacteria. The combination of enterocin AS-48 and *polyphosphoric acid* (an organic chemical compound) was significantly able to inhibit the growth of the population of *E. coli* O157:H7 in sprouted samples [15]. Application of a combination of bacteriocins and natural compound in foods could potentially be used as part of a hurdle technology that works synergistically and provides a better antibacterial activity [33]. However, future research requires to be elucidated to find the right concentration of combination, and proper extraction of Asam Sunti.

CONCLUSION

In this study, the in vitro test of bacteriocin alone can inhibit all MDR *E. coli* isolates, while the combination of bacteriocin and Asam Sunti extract was only able to inhibit three isolates.

The combined application of bacteriocin and Asam Sunti extract on fresh lettuce had the best reduction in total bacteria in isolates LL1.2 (bacteriocin only) and LL3.11 (ratio 2:1) by 97%. The best decrease in MDR *E. coli* density was found in isolate LL1.2 by 94% with bacteriocin immersion treatment, Asam Sunti extract, and a combination of 1:2 ratio. From this research, we can know that fresh lettuce contains MDR *E. coli* and can be controlled with bacteriocins and natural compounds. Further research is needed to optimize the combination by considering variations in temperature, pH, and other growth factors, to produce products for washing fresh vegetables.

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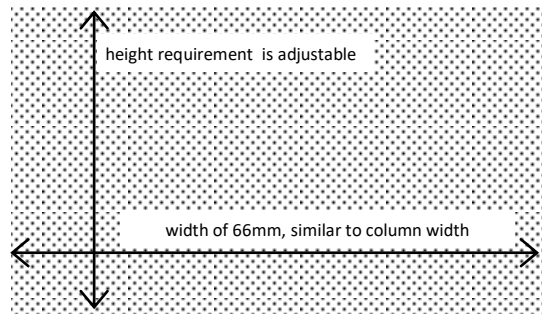


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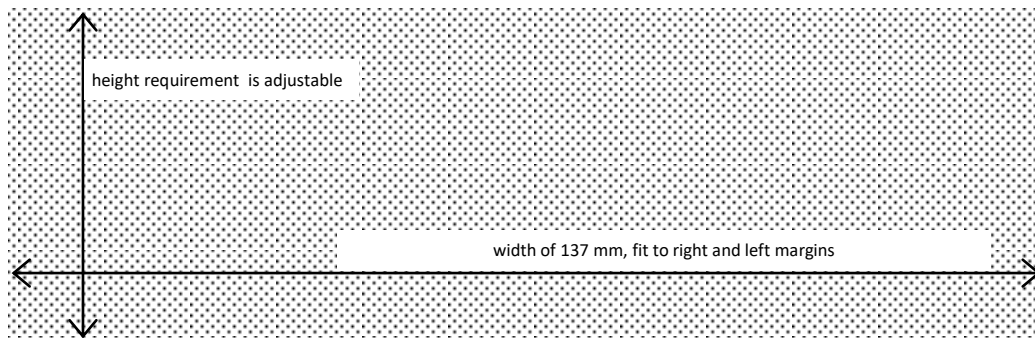


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- [4].Syafi'i, M., Hakim, L., dan Yanuwiyadi, B. 2010. Potential Analysis of Indigenous Knowledge (IK) in Ngadas Village as Tourism Attraction. pp. 217-234. In: Widodo, Y. Noviantari (eds.) Proceed-ing *Basic Science National Seminar 7* Vol.4. Universitas Brawijaya, Malang. (Article within conference proceeding)
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CONCLUSION (Calibri 10 Bold, Left, Capslock)

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ACKNOWLEDGEMENT (Calibri 10 Bold, Left, Capslock)

This section describes gratitude to those who have helped in substance as well as financially.(Calibri 10 Justify)

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