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## 4 Effectiveness of using of brown algae alginate to immobilize the indigenous bioremediation bacteria for reducing waste water from shrimp culture

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**Abstract.** *Bacillus coagulans* T1.2, an indigenous bacterium from shrimp ponds in East Lampung, has been found to reduce total ammonia nitrogen (TAN). The purpose of this study was to know the effectivity of immobilization of bacterial by sodium alginate from marine brown algae to reduce wastewater pollution from shrimp culture. The brown algae, *Sargassum* sp. and *Padina* sp., were collected from Pesisir Barat and Ketapang beach, Lampung. Alginate was extracted from both *Sargassum* and *Padina* by alkali method. The bacterial immobilization beads were made by mixed *Bacillus coagulans* suspension with alginate (1:3 v/v) and formed beads by 1,5 ml syringe without needle. In the in vitro study, the immobilized bacteria were submerged in artificial wastewater (2 beads ml<sup>-1</sup>) and incubated for 20 days. The control group includes the same bacteria without immobilization. The TAN was measured and the viability of bacteria was evaluated after the incubation period. The results showed that using alginate from *Sargassum* sp. and *Padina* sp. as a matrix of immobilization indigenous bacterial *Bacillus coagulans* effectively and significantly reduce the content of Total Ammonia Nitrogen (TAN) in wastewater. The viability of bacteria immobilized with the alginate of *Sargassum* sp. and *Padina* sp. better than the bacterial treatment without immobilization.

### 7 1. Introduction

In the last five years, the national shrimp production volume has shown a positive growth trend with an average growth of 15.7% [1]. Shrimp production that occurs continuously will cause problems of decreasing environmental carrying capacity. This happens because biochemical processes involving suspended particles, inorganic nitrogen, and phosphorus nutrients in the cultivation environment will have a direct impact on the content of ammonia, nitrite, hydrogen sulfide (H<sub>2</sub>S) compounds, and carbon compounds that are toxic for cultivation system [2].

Bioremediation is one way to overcome these problems. The utilization of bioremediation bacteria derived from external products is not effective to reduce organic waste in shrimp ponds due to unequal environmental conditions. Indigenous bacteria *Bacillus coagulans* are expected to accelerate the degradation process of pond waste because the habitat of these bacteria is same as current environmental conditions. Based on previous research, *Bacillus coagulans* bacteria has the potential as a



bioremediation agent. This bacterium is not pathogenic and can reduce levels of Total Ammonia Nitrogen (TAN) in vitro [3].

Storing isolates for a certain time can cause a decrease in bacterial bioactivity so that their potential is not optimal. Technology improvement is needed to increase the activity of these bacteria, a potential alternative is to improve its performance through immobilization using sodium alginate. The use of sodium alginate was chosen because its presence is easily obtained from the extraction of brown seaweed *Sargassum* sp. and *Padina* sp. which are plentiful in the coastal area of Lampung. Utilization of this type of brown seaweed as a source of alginate is expected to support the potential of *Bacillus coagulans* to optimally degrading TAN.

This study aimed to analyze the effectiveness of immobilization of indigenous *Bacillus coagulans* bacteria to reduce TAN using sodium alginate from *Sargassum* sp. and *Padina* sp.

## 2. Methods

### 2.1. Algae Collection

This study used the brown algae *Sargassum* sp. and *Padina* sp. obtained from coastal areas in Lampung Province. The *Sargassum* sp. was obtained from Pesisir Barat beach, and *Padina* sp. was obtained from Ketapang Beach.

2.2. *Design Experimental.* This study was conducted using four treatments of immobilized bacteria to degrade TAN in Sewage medium, both for sodium alginate from *Padina* and *Sargassum*. The four treatments were control group that was without adding sodium alginate and bacteria (A), adding sodium alginate without bacteria (B), adding immobilized bacteria using sodium alginate (C), and adding bacteria without immobilization (D).

### 2.3. Alginate Extraction and Characterization.

2.3.1. *Alginate Extraction.* Alginate extraction was carried out on *Sargassum* sp. and *Padina* sp. which has a moisture content of <15%. The alginate extraction process was preparing 100 g of *Sargassum* sp. and *Padina* sp. powder then soaked in distilled water (1:10). After that, added 5% Na<sub>2</sub>CO<sub>3</sub>/50μM EDTA for 24 hours. The immersion results were filtered and added with 0.13 M KCL. Then, precipitation was carried out with 96% cold ethanol (1:1) while vigorously stirring until homogeneous and allowed to stand for 24 hours. The precipitation results were centrifuged at 3,500 rpm for 5 minutes. The results of the alginate extraction were then collected and dried in an oven at 60 °C overnight [4].

2.3.2. *FTIR Analysis.* Extraction results of *Sargassum* sp. and *Padina* sp. the functional groups were analyzed using the FTIR (Fourier Transform Infra-Red) Spectroscopy method. The test compared extracted alginate from *Sargassum* sp. and *Padina* sp. with commercial alginate. This test was weighing of 1 mg samples, then mixed with 100 mg of KBr. Then pressed for ± 10 minutes in 8-10 psi, until a thin pellet is obtained. The pellet was inserted into the cell holder and the spectra were made for analysis [5].

### 2.4. Bioremediation Bacteria Culture and Immobilization

The immobilization followed an entrapment method using *Bacillus coagulans* bacteria. *Bacillus coagulans* is an indigenous bacterium from traditional tiger shrimp ponds in Mulyosari Village, Pasir Sakti District, East Lampung Regency, Lampung Province. These bacteria have the potential as biodegradation agents to reduce TAN [3].

*Bacillus coagulans* bacteria were cultured in 1000 mL liquid SWC (Sea Water Compete) medium. The SWC medium consists of 5 g bacto peptone, 1 g yeast extract, 3 ml glycerol, 75% distilled water, and 25% sterile seawater. And then the bacteria were incubated for 24 hours at 30 °C. These bacterial culture was mixed with Na-Alginate solution extracted from *Sargassum* sp. (5%) for *Padina* sp. (10 %) in a ratio (1:3) (bacteria:Na-alginate(v/v)).

The mixture was printed using a syringe 0.1 mL and dripped it drop by drop in a container that already contains a 0.2 M  $\text{CaCl}_2$  solution for the gelatinization process to form alginate beads (balls). The alginate beads were stored in the refrigerator for 6 hours. After that, the alginate beads were washed with physiological NaCl and put in 150 mL of sewage medium [6].

### 2.5. Sewage medium assay

Sewage medium is a TAN simulation medium consisting of 3.5 g  $\text{K}_2\text{HPO}_4$ ;  $\text{KH}_2\text{PO}_4$  0.7 g;  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  0.1 g;  $\text{NaHCO}_3$  0.5 g;  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  0.014 g;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  0.18 g;  $\text{NH}_4\text{Cl}$  0.1 g; EDTA 0.2 g [7]. The using of immobilized *Bacillus coagulans* bacteria 1 – 2 balls/mL into sewage medium. Incubation was carried out at 24°C using a shaker (100 rpm) for 20 days [8].

### 2.6. Total ammonia nitrogen (TAN) reduction

Analysis of TAN content was carried out at the beginning and end of the study. The steps in the analysis of TAN content include: standard ammonia solution prepared with a concentration of 0.1; 0.2; 0.4; 0.6; 0.8; 1 mg/L. This standard solution is used to determine the standard curve for ammonia. From each filtered sample, 10 mL was taken, and then 0.5 mL of phenol solution ( $\text{C}_6\text{H}_5\text{OH}$ ) was added and homogenized. After that, 0.5 ml of 0.5% sodium nitroprusside ( $\text{C}_5\text{FeN}_6\text{Na}_2\text{O}$ ) solution and 1 ml of oxidizing solution were added. The oxidizing solution consisted of an alkaline citrate solution ( $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$ ) and 5% sodium hypochlorite ( $\text{NaClO}$ ). The samples were allowed to stand for 1 hour (28–31°C). The absorbance was measured using a spectrophotometer with 640 nm wavelength. The absorbance value was entered in standard curve formula to determine TAN content in sewage medium [9].

### 2.7. Statistical Analysis

Statistical analysis was tested with One Way ANOVA using the SPSS 22.0 program to determine the level of difference between treatments. If there was a difference between treatments, then further test of LSD (Least Significant Difference) with 95% confidence interval.

## 3. Results and Discussion

### 3.1. Yields

The yield is a percentage between the weight of the final product and the weight of dry samples used during extraction. Determination of yield is useful for knowing the results of *Sargassum* sp. and *Padina* sp. extraction. The results showed that the yield of Na-Alginate from 100 g *Sargassum* sp. powder extraction was 19.80%. This value was lower than previous study that it reached 40.34% [10], but it higher than several previous studies 17.39% [11], and 12.88% [12].

Meanwhile, the yield of 100 g of *Padina* sp. extraction reached 14.44%. This value was lower than the results of previous studies, which reached 25% of the dry weight of *Padina* sp. extracted [13]. The factors that affect the yield of alginate include: type of seaweed used, conditions of seaweed grow place or habitat (light intensity, wave size or current, water-nutrition, etc.), climate, and the extraction method used, as well as how to handle it [14].

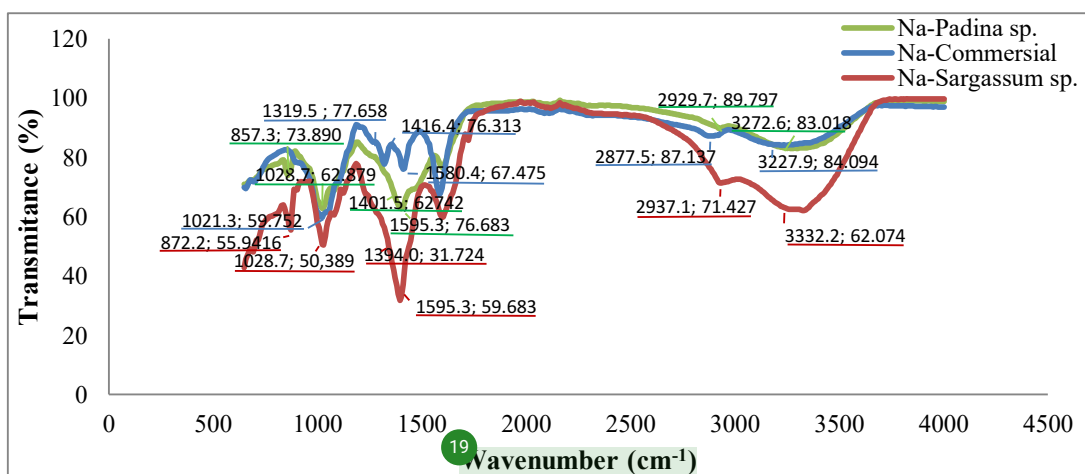
In the extraction process, using  $\text{Na}_2\text{CO}_3$  solvent was considered to separate cellulose and alginate in brown algae cells. The concentration of  $\text{Na}_2\text{CO}_3$  used during the extraction process also affects the yield produced. A high concentration of  $\text{Na}_2\text{CO}_3$  can produce more yield [15].

### 3.2. FTIR (Fourier Transform Infra-Red) Spectra Analysis

The FTIR test was carried out at 4000 – 650  $\text{cm}^{-1}$  wave length on 32 scanned samples and 4  $\text{cm}^{-1}$  resolution. The test was conducted to determine the presence of chemical bonds in organic compounds in *Sargassum* sp. and *Padina* sp. extract. The results of functional group analysis were compared with data from various references to confirm the presence of functional groups representing a sodium alginate bond at a certain wavelength (Table 1).

**Table 1.** Functional group of alginate based on FTIR spectra.

	Wavelength (cm <sup>-1</sup> )			
Na-Alginate <i>Sargassum</i> sp. Extract	Na-Alginate <i>Padina</i> sp. Extract	Na-Alginate Commercial	Functional Group Interpretation	Wavelength Reference (cm <sup>-1</sup> )
<b>3332.2</b>	3272.6	3227.9	Hydroxyl Group (O-H)	3200 – 3500 <sup>[16]</sup>
<b>2937.1</b>	2929.7	2877.5	Aliphatic Group (C-H)	2850 – 3000 <sup>[16]</sup>
<b>1595.3</b>	1595.3	1580.4	COO-asymmetric	1620 <sup>[17]</sup>
<b>1394.0</b>	1401.5	1416.4	COO-symmetric	1410 <sup>[17]</sup>
<b>1028.7</b>	1028.7	1319.5	Manuronic Acid and Carboxyl Group (C-O)	817,82 – 1315 <sup>[18]</sup> and 1000 – 1300 <sup>[16]</sup>
	857.3	1021.3	Guluronic Acid	817,82 – 1029 <sup>[18]</sup>



**Figure 1.** FTIR spectra of sodium alginate.

Vibration spectra at 3200 – 3500  $\text{cm}^{-1}$  indicate the presence of a hydroxyl group (O–H) bonded to hydrogen, vibrations at 2850 – 3000  $\text{cm}^{-1}$  indicate the presence of aliphatic compounds, and at 1000 – 1300  $\text{cm}^{-1}$  indicate the presence of a carboxyl group (C–O) [16]. Vibration spectra at 1620  $\text{cm}^{-1}$  indicated the presence of COO-asymmetric and at 1410  $\text{cm}^{-1}$  indicated the presence of COO-symmetric [10]. Vibration spectra at 817.82–1315  $\text{cm}^{-1}$  indicated the presence of mannuronic acid, and at 817.82–1029  $\text{cm}^{-1}$  indicated the presence of guluronic acid [18]. The presence of mannuronic acid and guluronic acid means that the sample contains alginate compounds [19].

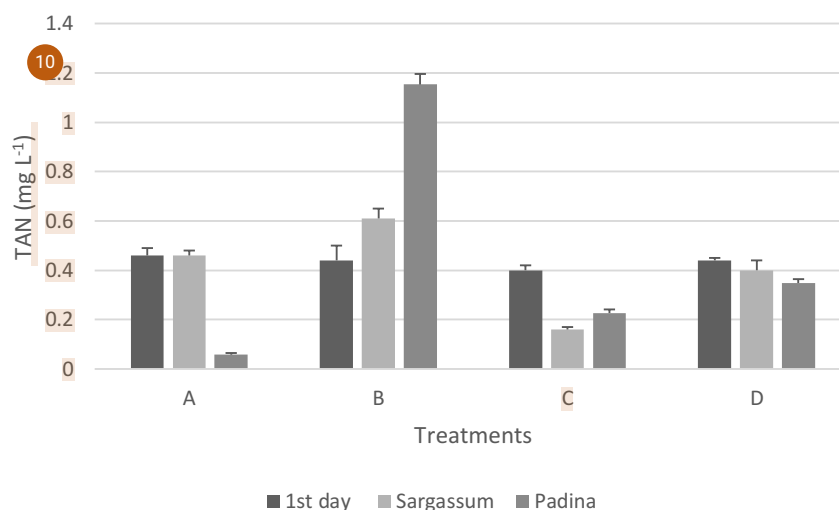
The FTIR test results on samples of *Sargassum* sp., *Padina* sp., and commercial alginates (**Figure 1.**) showed slightly different spectral patterns. The sodium alginate extracted from *Padina* sp. has a lower spectrum pattern than sodium alginate extracted from *Sargassum* sp. and commercial alginates (**Figure 1**). This happens because other functional groups are also filtered during the sample purification process. However, the extraction results from *Sargassum* sp. and *Padina* sp. are still identified as sodium alginate because it has met the standard by having 3 spectral peaks consisting of hydroxyl groups, COO-asymmetric, and COO-symmetric [17].

### 3.3. Analysis TAN (Total Ammonia Nitrogen)

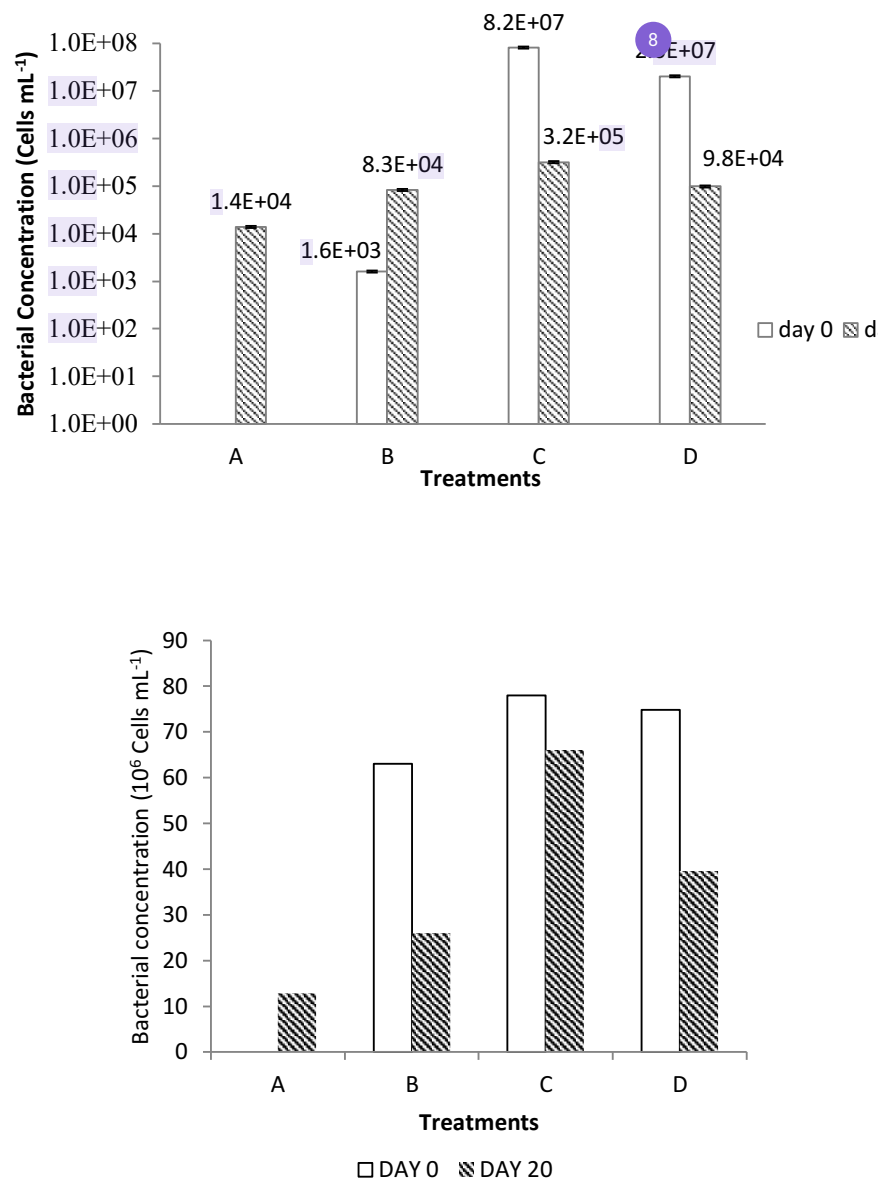
Analysis of TAN content was carried out to determine the effectiveness of immobilization of *Bacillus coagulans* bacteria using sodium alginate from *Sargassum* sp. and *Padina* sp. The TAN content at the beginning treatment was 0.45 – 0.48 mg/L, then decreased until the end of the study. The decrease TAN content occurs because *Bacillus coagulans* bacteria can utilize TAN compounds as metabolism for their growth [3].

The results showed the utilization of immobilized bacteria *Bacillus coagulans* with sodium alginate from *Sargassum* sp. and *Padina* sp. can increase the activity of bacteria better than free cells. This is known based on the decrease in TAN content in both treatments. Both treatments had a higher reduction in TAN levels than *Bacillus coagulans* without immobilization (**Figure 2**). The utilization of immobilized bacteria can absorb nitrogen better than free cells [20].

The highest decrease TAN content occurred in immobilization treatment of *Bacillus coagulans* bacteria using sodium alginate with *Sargassum* sp. The decrease TAN content in this treatment reached 60% (**Figure 2**). At the end of study, the results of One Way ANOVA test showed that there were differences in concentration of TAN in each treatment ( $\alpha < 0.05$ ). The results of LSD test showed that each treatment had significantly different results. Thus, assumed that immobilization of *Bacillus coagulans* bacteria using sodium alginate matrix from *Sargassum* sp. and *Padina* sp. can to increase activity of these bacteria to reduce TAN significantly.



**Figure 2.** TAN Concentration in Sewage Medium: (A) Control (without adding sodium alginate and bacteria), (B) Adding sodium alginate (C) Adding immobilized bacterial using sodium aginate (D) Adding bacteria without immobilization. The values is average of TAN concentration  $\pm$  standard deviation (SD).



**Figure 3.** Viability of bacteria in Sewage medium which treated by sodium alginate immobilization matrix from *Sargassum* (A) and *Padina* (B); A: Control, B: Immobilization of *Bacillus coagulans* with Na-*Sargassum* sp., C: Immobilization of *Bacillus coagulans* with Na-*Padina* sp., D: *Bacillus coagulans* without immobilization.

### 3.4. Bacterial Viability

The bacterial viability test was performed to determine the density of bacteria that grew in sewage medium. This method has counted the colonies that grow on agar medium. The bacterial counts were performed on free cells detached from alginate beads. During the research process, bacteria were released from the alginate beads and counted as free cells. The cells released from the alginate beads

occurred due to the diffusion of nutrients in alginate beads, causing a decrease in density and rigidity of the gel matrix. The use of sodium alginate concentration also affects the strength of matrix protection in holding cells out of the alginate bead [21].

Figure 3 showed that immobilization of *Bacillus coagulans* bacteria using sodium alginate from *Sargassum* sp. had the highest viability and was more stable than other treatments. Meanwhile, in the treatment of immobilized *Bacillus coagulans* using sodium alginate from an extract of *Padina* sp. and *Bacillus coagulans* without immobilization occurred decrease in bacterial viability until the end of the study. The decreased viability of bacteria in a medium can be caused by a reduction source of nutrients in the media, and saturation of the growth of bacteria is achieved [22].

Some factors cause decreased bacterial viability, including availability and amount of bacteria concentration in the samples. Bacterial growth biomass can also be affected by nutrients in the media. The lack of nutrients in the media for the needs of microorganisms causes the metabolism of *Bacillus coagulans* bacteria is not optimal so that the bacterial density decreases [23].

## 11. Conclusion

Based on the results, it was concluded that the immobilization of endogenous bacterium *Bacillus coagulans* using sodium alginate from *Sargassum* sp. and *Padina* sp. extract effective to reduce the content of Total Ammonia Nitrogen (TAN). The treatment showed that the viability of bacteria was better than Indigenous *Bacillus coagulans* without immobilization.

## Acknowledgment

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