Anti-dengue potential of bioactive protein from exophytic bacteria that are symbiotic with brown algae Sargassum sp.

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Abstract. Symbiont bacteria of algae are bioactive metabolite sources with potential as medicinal raw materials. This study aims to find out the anti-dengue potential of a protein fraction isolated from Enterobacter agglomerans SB 5(1) as the symbiont of brown algae Sargassum binderi collected from Lae-Lae island, South Sulawesi. These extracellular and intracellular fractions were isolated by ammonium sulphate fractionation at saturation levels of 0-20 %, 20-40 %, 40-60 %, and 60-80 %. The protein was purified by dialysis method using cellophane bag. Toxicity was tested by BSLT method using shrimp larvae of Artemia salina, Leach. Cytotoxicity test against vero cells infected with dengue virus DEVN-2 was performed by MTT method. Study findings indicate that intracellular protein fraction from E. agglomerans SB 5(1), a symbiont of brown algae Sargassum binderi, showed the presence of bioactive protein having strong toxicity with LC₅₀ of 48.67 µg/mL. Anti-dengue activity toward vero cells indicates inhibition percentage and CC₅₀ value of 70% and 260.37 µg/mL, respectively, therefore it had no potential as anti-virus dengue agent. In future studies, it is recommended to perform hydrolysis of protein compound from symbiont bacteria of Sargassum sp. to explore other peptide compounds with more potential as anti-dengue agents.

1. Introduction
The ocean involving 90% of the biosphere covers around 70% of the world's surface. Species of marine biota makeup about a half portion of worldwide variety and have been widely investigated in the past decade as a source of potential new bio active compounds as therapeutic raw materials [1]. Marine organisms such as algae are very potential in producing bioactive compounds that have very diverse physiological functions and can be developed as a therapy for various diseases [2]. Algae are one of the sea eukaryotes which are rich in symbiont bacteria on the surface and in algal tissue. Algal
symbionts produce bioactive compounds with unique and very diverse characteristics [3]. Marine eukaryotes including macroalgae associate and depend on bacteria for growth, development, nutrient supply and protection against predatory attacks [4]. Bacterial isolates from the surface part of algae (exophytic bacteria) as well as inside the tissue (endophytic bacteria) was found in 149 macroalgae associated with bacteria (36 from Chlorophyta, 46 from Phaeophyceae, 55 from Rhodophyta, and 12 algae not yet determined) in the past 40 years [5]. The potentials of Indonesia’s marine biota have motivated scientists and producers of the world's bioactive compounds to begin looking at the ocean as a potential source. Algae is one of the most potential marine natural resources in Indonesia [6]. The protein content between algae species varies, marine algae contain 5-15%, while red and green algae contain 10-47% protein from dry weight.

Symbiont bacteria have almost the same ability as their hosts in producing bioactive compounds, including proteins and peptides [7]. The ability of the symbiont bacteria to produce bioactive compounds is a very big opportunity in the supply of raw materials for drugs and nutraceuticals. A large number of algae exploitation without cultivation will certainly cause the extinction of certain algae in their habitat. Therefore, the use of algae symbiont bacteria is one of the best alternatives. In addition, breeding or bacterial culture of symbiont can be implemented easily in very large quantities, where growth and productivity can be easily controlled and production time is shorter. Thus, the use of symbiont bacteria as a source of medicinal raw materials is estimated to be economically more efficient.

On the other hand, Dengue Hemorrhagic Fever (DHF, dengue) is a disease transmitted by Aedes aegypti mosquito caused by dengue virus infection (DENV). An estimated 390 million dengue infections occur each year, of which 96 million manifests clinically. Prior to 1970, only nine countries encountered a dengue fever pestilence. At present, dengue fever is endemic in more than 100 countries, especially in tropical and sub-tropical countries [8]. The number of dengue cases reported increased from 2.2 million in 2010 to 3.2 million in 2015. There are four serotypes of dengue virus, DENV-1, -2, -3, and -4, which are genetically and antigenically different, although each serotype presents a series of similar clinical signs and symptoms during the period of infection [9]. Dengue fever remains a threat to the community regionally, nationally, and locally, especially in South Sulawesi, Indonesia. Until now there has been no effective DHF drug as an anti-dengue agent, especially from natural products. Specific therapy with effective anti-viral drugs has not been found to date, and the dengue virus vaccine is commercially unavailable. A number of researchers report a direct association between the viral load of dengue in the blood during the viremia phase and the severity of dengue [10]. Therefore, decreasing viral load using effective antiviral compounds can prevent complications of Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) [11]. In this regard, it is very necessary to conduct research in order to search for alternative drugs for dengue disease, especially from natural materials that are renewable and environmentally friendly such as protein compounds from symbiotic marine algae which have anti-dengue activities. Algae are known to be one of the sea eukaryotes which are rich in symbiotic bacteria [7]. From the results of several studies, it is known that symbiont bacteria of marine algae contain thousands of chemical compounds that have the potential as drugs with very diverse characteristics and unique physiological properties and functions [3,12]. Sargassum vulgare marine algae have been reported for antiviral screening and tested for cytotoxicity activity in Huh 7.5 cells giving an MNTD (Neutral Maximum Non-dose-Based on MTT and Neutral Red Assays) value of 62.5 µg/mL [13].

So far there is no research data that explore groups of protein compounds or peptides from algae-symbiont bacteria as raw material for anti-dengue. Proteins from the algae-symbiont bacteria E. agglomerans SB 5 (1) were isolated using the ammonium sulphate fractionation method followed by dialysis, then tested for toxicity and cytotoxic properties with each BSLT and MTT method. The results showed that intracellular protein fraction from E. agglomerans SB 5 (1), a symbiont of brown algae Sargassum binderi, contained bioactive protein compounds that had strong toxicity with LC_{50} values of 48.67 µg/mL. Anti-dengue activity against vero cells showed a percent inhibition and the CC_{50} value of 70% and 260.37 µg/mL respectively, so it had no potential as anti-dengue compound.
agents. Further studies will be carried out on partial hydrolysis of protein compounds to explore some types of peptide bioactives which have more potential as future anti-dengue agents.

2. Materials and methods

2.1 Materials

The materials used in this study were brown algae (Sargassum binderi and Sargassum polycystum, Egg of Artemia salina, Leach. vero cells, DENV-2 virus, DMEM media, 1% (w/v) L-glutamine, Nutrient Broth media (NB), Nutrient Agar (NA) media, BSA (Bovine Serum Albumine), Physiological NaCl, 70% alcohol, buffer A (Tris (hydroxymethyl) amino methane 0.1 M pH 8.3; NaCl 2 M; CaCl₂ 0.01 M, β-mercaptoethanol 1%, Triton X-100 0.5%), buffer B (Tris (hydroxymethyl) amino methane 0.1 M pH 8.3; NaCl 0.2 M; CaCl₂ 0.01 M), buffer C (Tris (hydroxymethyl) amino methane 0.01 M pH 8.3; NaCl 0.2 M; CaCl₂ 0.01 M), Lowry A (Phophotungstat-phosphomolybdic acid solution with distilled water 1: 1), Lowry B (Na₂CO₃ 2%; NaOH 0.1 N; CuSO₄.5H₂O 1% ; 2% potassium tartrate), ammonium sulphate, cellophane bags, universal pH paper, sea water, filter paper, cotton, aquades, spiritus, aluminum foil, cling wrap, and tissue roll.

2.2 Instrumentations

The tools used in this study include analytic balance, magnetic stirrer, thermo cycle PCR machine, electronic 20D+ Spectrometer, cold centrifuge, incubator, autoclave, oven, case, micropipette, tweezers, pH meter, refrigerator, water bath, mortar, petri dish, inoculation needle, bunsen, shakers, sonicators, vortices, spray bottles and glassware commonly used in laboratories.

2.3 Procedures

2.3.1 Sample Preparation

Algae samples that have been collected were then cleaned from the dirt attached and stored in a cool box. Next, the sample was refreshed in the NB media.

2.3.2 Sample Refreshment in NB Media

2.3.2.1 Surface Part of Algae

Fifteen grams of algae samples were rinsed with 30 mL of sterile sea water. Then the rinsed water was added into 30 mL NB media and then shaken using a shaker at room temperature for 24 hours.

2.3.2.2 Internal Part of Algae

About 15 grams of algae samples were rinsed with 30 mL of sterile sea water. Then the rinsed algae were crushed until smooth using a mortar and suspended with sterile sea water. The suspension was then added into 30 mL NB media and shaken using a shaker at room temperature for 24 hours.

2.3.2.3 Isolation of Symbiont Bacteria that Produce Anti-dengue Compounds

As much as 1 mL of samples that have been refreshed on NB media were added to a test tube containing 9 mL of sterile sea water. A multilevel dilution of 10⁻¹ to dilution of 10⁻⁵ was carried out. After that, each dilution was grown on NA media at 37°C for 2 × 24 hours. Following incubation, stable colonies were picked and scratched several times on the incubated NA media to obtain a single colony.

2.3.2.4 Selection of Bacteria Isolates that Produce Anti-dengue Compounds

Selection of isolates that produce anti-dengue compounds is carried out semiquantitatively, by scraping or dripping isolates on the surface of NA media. Then incubated for 1 x 24 hours at 37 °C. Bacteria isolates that produce LC₅₀ values below 1000 µg/mL with stable toxicity after three replications were selected for further research.
2.3.2.5 Identification of Bacteria Isolates that Produce Anti-dengue Compounds
Bacteria isolates which produce LC<sub>50</sub> values below 1000 µg/mL with stable toxicity after three replications were identified for their bacterial species by microbiological, biochemical and molecular analysis of 16S RNA.

2.3.2.6 Determination of Bioactive Protein Optimum Production Time
The bacterial isolates that have been identified were taken 2-3 loops and inserted into Erlenmeyer containing 100 mL of sterile NB media. Then the culture was incubated at 37°C on a shaking machine with a speed of 200 rpm for ± 4 days to determine the optimum production time of the protein. Then sampling was done every 12 hours to measure the optical density (OD<sub>600nm</sub>) and levels of the protein of the bacterial isolate culture.

2.3.2.7 Production and Extraction of Bioactive Protein with Potential as Anti-dengue
After the optimum production time of the protein was established, then the production in large quantities (volume) was performed for around 2000 mL under these optimal conditions. The cell fragments were then centrifuged at 5,000 rpm, 4°C for 30 minutes to obtain a crude extract of intracellular protein and then stored in the refrigerator before the toxicity test and subsequent purification process.

2.3.2.8 Fractionation and Dialysis
Crude extracts from extracellular and intracellular proteins were fractionated using ammonium sulphate at the percent saturation levels: 0-20%, 20-40%, 40-60%, and 60-80% following by dialysis with the cellophane bag (Sigma).

2.3.2.9 Determination of Protein Concentration
Determination of protein content in each fraction used the Lowry method with bovine serum albumin (BSA) as a standard solution [14].

2.3.3 Anti-dengue activity test
2.3.3.1 Toxicity test of protein compound for anti-dengue screening
Toxicity tests were carried out using the Brine Shrimp Lethality (BSLT) method according to [15]. A total of 4 mg of protein samples were dissolved in a 0.1 M Tris-HCl buffer pH 8.3 then diluted into several concentration variations (400; 200; 100; 50; 25; 12.5; 6.25; 3.13; 1, 56; 0.78 µg/mL) using sterile sea water. The solution was inserted into the vial as much as 5 mL. About ten shrimp larvae of Artemia salina, Leach were inserted into each sample vial using a transfer pipette. The viable nauplii can be calculated macroscopically on a pipette with a light background. The dry yeast suspension (3 mg in 5 mL sterile sea water) was added to each sample vial as a nutritional source for shrimp larvae of A. salina, Leach. Vials were placed under the light. Living nauplii was calculated using a magnifying glass after 24 hours, and the percentage of dead nauplii per sample was calculated. In cases where there are dead nauplii in the control, the data was collected using the Abbot formula 1 as follows:

\[
\% \text{ Mortality} = \frac{\Sigma \text{test larvae were dead} - \Sigma \text{control larvae dead}}{\Sigma \text{test larvae}} \times 100\%
\]  

The LC<sub>50</sub> was determined from the data of % mortality based on the probit analysis described by [15].

2.3.3.2 Anti-dengue test for protein compounds from symbiont bacteria of algae Sargassum, sp.
Cytotoxicity test of protein compounds from the isolated bacterial symbiont of algae *Sargassum*, sp. against vero cell was carried out to determine the cytotoxic effects of these compounds on vero cells. Cytotoxic tests were carried out using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. Cell line (vero cell) was grown on DMEM (Gibco) medium with added nutrients in the form of 10% Fetal Bovine Serum (FBS), 1% (w/v) L-glutamine, which was incubated at 37°C and 5% CO2. After reaching 70% of cell density (confluent), vero cells with a density of 10^4 cells/well were distributed into a well (96 well plate) and incubated in a CO2 incubator for 24 hours at 37°C and 5% CO2 gas. After that the vero cells were infected with the DENV-2 virus as a treatment group, then F4 protein fraction (60-80%) of the concentration series was added, namely 100, 50, 25, 12.5, 6.25 ppm each with 3 replications. Negative control was performed equally but was not infected with the DENV-2 virus. Calculation of the percentage of cell viability used the following formula.

\[
\text{% Cell viability} = \frac{\text{Test Absorbance Value-Medium Absorbance Value}}{\text{Control Absorbance Value-Medium Absorbance Value}} \times 100\% \quad (2)
\]

The CC50 value was determined from the % inhibition based on the probit analysis described by [16].

3. Results and discussion
3.1 Isolation of symbiont bacteria producing anti-dengue protein compound
In this study, isolation of symbiont bacteria from two types of brown algae *Sargassum* sp. collected from the waters of the island of Lae-Lae, South Sulawesi, Indonesia was conducted. From the two types of brown alga, 16 bacterial isolates were obtained at a stable temperature of 37°C, namely 6 isolates from the internal part of the algae tissue and 10 isolates from the algae surface (Tables 1 and 2).

To obtain anti-dengue protein compound isolates, a suspension with a greater number of microbes was needed so that existing bacteria will compete with each other in order to grow optimally. Isolates producing anti-dengue protein compounds are characterized by strong toxicity through the initial screening of BSLT tests on shrimp larvae *A. salina*, Leach, where activity level criteria is based on [17], where a substance is said to be active or toxic if the LC50 value is smaller than 1,000 μg/mL for an extract and smaller than 30 μg/mL for a pure compound.

3.2 Selection of isolates producing anti-dengue protein compounds
To determine the potential of algae-symbiotic bacterial isolates as a producer of anti-dengue protein compounds, BSLT was tested against *A. salina*, Leach shrimp larvae for initial screening of toxicity as shown in Table 1.

**Table 1.** The results of calculation of LC50 value against shrimp larvae (*A. salina*, Leach) from several symbiont bacteria isolates from the internal part of algae tissue.

| No | Brown Algae Type | Isolate Code | Extracellular | Intracellular |
|----|------------------|--------------|---------------|---------------|
| 1  | *S. binderi*     | BSB 4(1)     | > 1000        | > 1000        |
|    |                  | BSB 4(2)     | > 1000        | > 1000        |
|    |                  | BSB 5(1)     | > 1000        | > 1000        |
|    |                  | BSB 5(2)     | > 1000        | > 1000        |
| 2  | *S. polycystum*  | BSP 2(1)     | > 1000        | > 1000        |
|    |                  | BSP 2(2)     | > 1000        | > 1000        |

*Data were averaged from 3 replications
Table 2. The results of calculation of LC$_{50}$ against shrimp larvae ($A.\ salina$, Leach) from several isolates of symbiont bacteria from the surface part of algae.

| No | Brown Algae Type   | Isolate code | Extracellular | Intracellular |
|----|--------------------|--------------|---------------|--------------|
| 1  | *Sargassum binderi*| SB 2(1)      | > 1000        | > 1000       |
|    |                    | SB 2(2)      | > 1000        | > 1000       |
|    |                    | SB 3(1)      | > 1000        | > 1000       |
|    |                    | SB 3(2)      | > 1000        | > 1000       |
|    |                    | SB 5(1)      | 148.59        | **58.39**    |
|    |                    | SB 5(2)      | > 1000        | > 1000       |
|    |                    | SB 5(3)      | > 1000        | > 1000       |
| 2  | *Sargassum polycystum* | SP 1(1)    | > 1000        | > 1000       |
|    |                    | SP 1(2)      | > 1000        | > 1000       |
|    |                    | SP 3(1)      | > 1000        | > 1000       |

* Data were averaged from 3 replications

Based on the results of bacterial isolates BSLT test, as many as 6 isolates of symbiont bacteria isolated from the internal part of algae tissue, all showed LC$_{50}$ values greater than 1000 $\mu$g/mL (Table 1), so that it was categorized as non-toxic means very weak potential as anti-dengue virus. Whereas for symbiont bacterial isolates isolated from surface part of algae, from 10 bacterial isolates, one isolate was found showed an LC$_{50}$ value of 58.39 $\mu$g/mL (Table 3) so that it was categorized as very toxic meaning having very high potential as an anti-dengue agent.

Based on the stability and representative values of LC$_{50}$ after three tests, then for the next study, SB 5 isolates (1) were isolated from the surface part of algae. (Figure 1) selected as bacterial isolates for identification and extraction of bioactive protein with the potential to produce anti-dengue compounds.

Figure 1. Isolate of SB 5(1) bacteria isolated from surface part of brown alga $S.\ binderi$. 

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3.3 Identification of isolates producing anti-dengue protein compound

Candidates of isolates of SB 5 bacteria (1) isolated from the inside of the brown algae *Sargassum binderi* tissue were further identified for the species. Identification was carried out by gram staining and simple biochemical tests including TSIA test (Triple Sugar Iron Agar), SIM (Sulfid Indol Motility), fermentation test of sugars, citrate, urea, and VP-MR ((Methyl Red-Voghr Proskaur). The results of species identification can be seen on Table 3.

Microscopic testing showed that SB 5 (1) bacterial isolate was Gram-positive as indicated by cells that were red on Gram staining. The bacterial cells were rod-shaped according to observation using a light microscope with 1000x magnification (data is not shown).

The results of biochemical tests with TSIA media on isolates of SB 5 bacteria (1) showed positive results. TSIA media is a medium that contains three types of sugar, namely glucose, lactose, and sucrose. The incubation results show that carbohydrates in the medium underwent fermentation. This was indicated by the change in colour of the media from pink to yellow. The bacteria did not produce H$_2$S because black deposits did not form but produced gas which was characterized by the rupture of the media at the bottom end of the test tube. Motility test on SIM media showed that these bacteria were motile which was marked by puncture marks widening on the media. The citrate test results showed that the bacteria had the ability to ferment citrate which was indicated by the change in color of the media from green to blue. While the urea test showed that the bacteria produced urease which can hydrolyze urea, characterized by the formation of red color.

The results of the fermentation process showed that the bacteria formed acid which was marked by changes in the color of the media and followed by the formation of gas bubbles in the Durham tube. The acid produced from the fermented form was mixed acid (methylene glycol) which was marked in yellow to red in the Methyl Red test. This bacterium was able to use butanediol fermentation through the Voges-Proskauer test by changing the yellow color to red.

| Table 3. The results of bacteria SB 5(1) isolate identification |
|---------------------------------------------------------------|
| **Test** | **Gram Staining** | **Gram Type** | **Rod** | **Negative** | **Results** |
|---------|------------------|---------------|---------|-------------|-------------|
| TSIA    | Slant            | +             |          |             |             |
|         | Butt             | +             |          |             |             |
|         | H$_2$S           | -             |          |             |             |
|         | Gas              | +             |          |             |             |
| SIM     | Indol            | -             |          |             |             |
|         | Motility         | +             |          |             |             |
| MR-VP   | MR               | +             |          |             |             |
|         | VP               | +             |          |             |             |
| Citrate |                 |               |          | +           |             |
| Urea    |                 |               |          | +           |             |
| Sugars  | Glucose          | +             |          |             |             |
|         | Lactose          | +             |          |             |             |
|         | Sucrose          | +             |          |             |             |
|         | Mannitol         | +             |          |             |             |

From several tests that have been performed both Gram staining and simple biochemical tests and supported by morphological and physiological characteristics of previous studies, the symbiont bacterial isolate showed the characteristics of *Enterobacter agglomerans*. This result was confirmed by molecular analysis using 16S RNA method (Figure 2). Thus this isolate was then named *E. agglomerans* SB 5 (1). Some studies also found the presence of symbiont bacteria from the
Enterobacter sp group in green algae and sponges which have antimicrobial activity and are toxic to shrimp larvae A. salina, Leach [18,19].

![Phylogenetic Tree](image)

**Figure 2.** Construction the phylogenetic tree of an isolate of SB 5(1) bacteria isolated from the surface part of brown algae S. binderi

3.4 Determination of bioactive protein optimum production time

The initial stage in the production of bioactive protein was the rejuvenation of E. agglomerans SB 5 (1) which has the strongest toxicity. The bacteria were grown on agar NA plate media, then 2-3 loops of colonies were pick and inoculated into Erlenmeyer containing 100 mL of sterile NB media. Then the culture was incubated at 37°C on a shaker for ± 4 days. Every 12 hours sampling was done to determine the optimum time of bioactive protein production by measuring the optical density (OD$_{600nm}$) and protein content at a wavelength of 600 nm (Table 4). In this study, measurements were performed for protein levels from the (extracellular) filtrate and the (intracellular) bacterial cells.

Based on the data obtained in Table 4, it shows that in the 0th to 36th hour there was an adaptation phase with an environment where an OD$_{600nm}$ value was irregular. In this phase the growth increased slowly. In the 36th to 60th hours, there was an increase in the number of bacteria. This is due to increased bacterial division because it has adapted to its environment and the nutrients contained in the media were sufficient for bacterial growth. Then the stationary phase occurred in the 60th to 72nd hours.
In the final stationary phase, the protein content was highest, both extracellular and intracellular proteins with protein content values of 1.462 mg/mL and 0.975 mg/mL respectively. This is due to the highest bacterial growth observed so that the secretion of protein is even greater. At the 84th hour until the 96th hour, there was a death phase, which was marked by a decrease in the number of bacteria. This is due to a decrease in the number of nutrients contained in the media.

Proteins secreted by bacteria cells, both extracellular and intracellular, show an increase along with the length of fermentation time. From the graph in Figures 3 and 4 an increase in extracellular protein levels from the 0th hour to the 60th hour was shown. At the 72nd hour the highest protein content was obtained at 1.462 mg/mL and at the 84th to 96th hour there was a decrease in protein secretion. This is in accordance with the observed bacterial growth rate where the highest bacterial growth was observed at 72nd hours so that the secretion of protein was even greater. So even with intracellular protein, the highest protein content was obtained at 72 hours at 0.975 mg/mL. From the
data obtained it can be seen that extracellular and intracellular proteins have the same optimum production time, but what distinguishes them was the level of protein produced where extracellular protein levels were higher than intracellular proteins.

**Figure 4.** The effect of fermentation time on intracellular protein production and *E. agglomerans* SB 5(1) cell growth

3.5 Extraction and isolation of bioactive protein

Extraction and isolation of bioactive proteins from isolates of *E. agglomerans* SB 5 (1) used modified procedure from previously reported methods [20]. The extraction and isolation of this protein were carried out at low temperatures (0-4°C) with the help of buffer A. This is because proteins are a very heterogeneous group of biomacromolecules. When outside the cell, the protein is very unstable and can be denatured at high temperatures. In addition, protein is also sensitive to acids or bases with high concentrations so that protein purification was carried out at low temperatures in a buffer and a certain pH [21].

**Table 5.** Distribution of extracellular and intracellular protein concentration each percentage saturation of ammonium sulphate fraction isolated from exophytic bacteria *E. agglomerans* SB 5 (1)

| No | Protein Type  | Protein Fraction | Volume of each fraction (mL) | Protein level (mg/mL) | Total protein (mg) |
|----|---------------|------------------|-------------------------------|-----------------------|-------------------|
| 1  | Extracellular protein | 0-20 %          | 9.0                           | 1.002                 | 9.018             |
|    |                | 20-40 %         | 9.0                           | 0.387                 | 3.483             |
|    |                | 40-60 %         | 8.5                           | 0.437                 | 3.715             |
|    |                | 60-80 %         | 8.5                           | 0.693                 | 5.891             |
| 2  | Intracellular protein | 0-20 %         | 10.5                          | 2.705                 | 28.424            |
|    |                | 20-40 %         | 9.0                           | 1.083                 | 9.747             |
|    |                | 40-60 %         | 9.0                           | 5.365                 | 48.285            |
|    |                | 60-80 %         | 8.5                           | 6.530                 | 55.505            |

Protein deposits from each of the F1, F2, F3, and F4 fractions were dissolved with an amount of buffer B, which has almost the same volume per fraction, then put in a cellophane bag. Cellophane bag which has been filled with protein fraction was put into a beaker glass containing a buffer C solution and then stirred with a magnetic stirrer to accelerate the protein purification process. The protein content of each fraction of both extracellular and intracellular proteins after dialysis can be seen in Table 5.
Table 5 shows that distribution of protein concentration in each fraction of ammonium sulphate percent saturation, F1 (0-20% fraction), F2 (20-40%), F3 (40-60), and F4 (60-80%). This happens because of differences in the solubility of proteins in the water so that the protein that precipitates is also different. Proteins that have small solubility in water precipitate earlier compared to proteins with higher solubility in water. The highest levels of extracellular protein were found in the 0-20% fraction (1.002 mg/mL), while the intracellular protein was found highest in concentration in the 60-80% fraction (6.530 mg/mL). From these data, it can be assumed that extracellular proteins are a type of protein less soluble in water while intracellular proteins were considerably more soluble in water.

3.6 Toxicity test with Brine Shrimp Lethality Test (BSLT)
In this study, the toxicity test was performed on the bioactive protein from the intracellular exophytic bacteria E. agglomerans SB 5 (1) against shrimp larvae Artemia salina, Leach. Table 6 shows that crude extract and bioactive protein from intracellular exophytic bacteria E. agglomerans SB 5 (1) has varying values. F0 fraction (crude extract), F1 (0 - 20% fraction) and F2 (Fraction 20 - 40%) are at non-toxic levels. While F3 (40 - 60% fraction) and F4 (60 - 80% fraction) are at the toxic interval. Protein fraction with the saturation of 20 - 40% (F2) gave a non-toxic response to shrimp larvae Artemia salina, Leach. with the highest LC50 value of 75,353.82 µg/mL. Protein fraction with the saturation of 60-80% (F4) gave a very toxic response to shrimp larvae Artemia salina, Leach with the lowest LC50 value of 48.67 µg/mL.

Table 6. LC50 values of dead shrimp larvae Artemia salina Leach against crude extract and bioactive protein fraction from intracellular part of exophytic E. agglomerans SB 5 (1)

| Protein Fraction | LC50 (µg/mL) | Toxicity   |
|------------------|--------------|------------|
| (F0) Crude Extract | 1,845.02     | Not Toxic  |
| (F1) Fraction 0 – 20% | 68,977.85    | Not Toxic  |
| (F2) Fraction 20 – 40% | 75,353.82    | Not Toxic  |
| (F3) Fraction 40 – 60% | 482.62       | Toxic      |
| (F4) Fraction 60 – 80% | 48.67        | Very Toxic |

3.7 Anti-dengue potential activity test for bioactive protein from exophytic bacteria toward vero cells infected with dengue virus DENV-2
Cytotoxicity test of bioactive protein fraction from E. agglomerans SB 5 (1) exophytic bacteria showed that the highest toxicity in BSLT test was F4 fraction (60-80%), followed by a cytotoxic test on vero cells. The test was carried out by the MTT method based on the change of tetrazolium salt into a colored product by the mitochondrial succinate dehydrogenase enzyme with the help of cellular NADH to evaluate their cytotoxicity of compounds. The percentage of living vero cells (cell viability) was calculated based on the ratio between the absorbance of the test compound treatment and vero cell control. The percentage of cell viability, percent inhibition, and CC50 values after administration of bioactive protein fraction F4 (60-80%) from intracellular exophytic bacteria E. agglomerans SB 5 (1) can be seen in Table 7.

Table 7. Anti-dengue potential results after the administration of 20 µg/mL bioactive protein fraction F4 (60-80%) from intracellular part of exophytic bacteria E. agglomerans SB 5 (1)

| Protein fraction | cell viability (%) | Inhibition (%) | CC50 (µg/mL) |
|------------------|--------------------|---------------|--------------|
| Control (-)      | 20                 | 50            | >1000        |
| (F4) Fraction 60- 80% | 40                | 70            | 260.37       |
Based on the conventions of some of the results of previous studies [22, 23] that the criteria for a natural substance to be said to have anti-dengue activity if cell viability is > 50%, percent inhibition > 90%, and CC₅₀ < 250 µg/mL. Based on these criteria it can be concluded that the bioactive protein fraction F4 (60-80%) of the intracellular exophytic bacteria E. agglomerans SB 5 (1) does not have the potential as an anti-dengue agent. Therefore, it is necessary to further explore the types of protein from the symbiont bacteria Sargassum, sp or carry out the partial hydrolysis process of protein compounds to obtain very bioactive peptides with smaller molecular size so that it is easier to penetrate the dengue virus that causes DHF.

4. Conclusion
From the study findings, it can be concluded as follows: (1) Sixteen isolates of bacteria with stable colony were successfully isolated from two types of brown algae consisting of 6 isolates from the internal part of algae tissue and 10 isolates from surface part of algae. (2) Bacteria with the highest toxicity value against the shrimp larvae of A. Salina, Leach were derived from surface part of brown algae Sargassum binderi and identified as E. agglomerans SB 5(1). (3) Intracellular protein fraction of symbiont bacteria of brown algae E. agglomerans SB 5(1) showed more potent toxicity than extracellular protein fraction. Anti-dengue activity against vero cells indicated inhibition percentage and CC₅₀ value of 70% and 260.37 µg/mL, respectively, therefore, it had no potential as anti-dengue agents. In future studies, it is recommended to perform hydrolysis of protein compounds from symbiont bacteria of Sargassum sp. to explore other peptide compounds with more potential as anti-dengue agents.

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