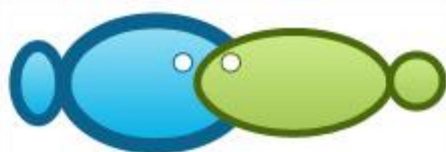

Screening, characterization, and identification of fucoidanase producing bacteria from *Sargassum polycystum*

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Abstract. Fucoidanase, an enzyme that catalyzes the cleavage of glycoside bonds between sulfated fucose residues in fucoidan, is found in various marine organisms, including bacteria, fungi, and invertebrates. However, there are few studies on the use of the symbiont *Sargassum polycystum* as a fucoidanase producer. This study aims to isolate, characterize, and identify fucoidanase-producing bacteria from *S. polycystum* morphologically, biochemically, and molecularly using 16S rDNA polymerase chain reaction (PCR). Isolation of bacterial symbionts was carried out by 2 methods, namely the spread plate and streak plate methods. The pure isolate was tested qualitatively and semi-quantitatively on media selection to see the clear zone produced after the administration of 10% cetyl pyridinium chloride (CPC) solution. Subsequent activity tests were conducted quantitatively, to determine the specific fucoidanase activity produced. The results showed that 4 isolates of symbiotic bacteria from *S. polycystum* had the greatest fucoidanase activity tested by the CPC method. The following isolates had the greatest fucoidanase activity index values: GSD (10.66±2.26), PTF (8.69±0.36), PSA (8.23±2.64), and GSB (7.92±0.11). The GSD isolate had the greatest activity of fucoidanase production at 48 and 72 h compared to the other 3 isolates, namely 0.0072 U mg^{-1} and 0.0083 U mg^{-1}, respectively. Morphological, biochemical, and molecular identification with 16S rDNA PCR found that the three isolates with the greatest fucoidanase activity were *Cytobacillus kochii* (GSD), *Bacillus cereus* (PTF), and *Brevibacterium sediminis* (PSA) bacteria with 99% similarity. The fucoidanase produced is expected to be used for industrial-scale enzyme production in Indonesia. In addition, the use of symbiotic bacteria in brown algae is expected to reduce the exploitation of *S. polycystum* and contribute to the preservation of marine ecosystems.
Key Words: bacterial symbiont, enzyme, fucoidan, 16S rDNA, *Sargassum polycystum*.

Introduction. Fucoidan is a complex polysaccharide in the structure of the cell wall of brown seaweed, which contains mostly L-fucose sulfate with small amounts of monosaccharides such as galactose, glucose, xylose, and uronic acid. Various chemical structures found in fucoidan are linked by glycosidic bonds -(1,2), -(1,3), -(1,4), and sulfate groups (Lim et al 2016). The chemical structure and molecular weight of fucoidan vary greatly depending on the type, harvest season, and climatic conditions of the brown seaweed (Wang et al 2019). Varied molecular weights can affect the bioactivity of fucoidans as antioxidants (0.8-10 kDa) (Yuan & Macquarrie 2015), anticoagulants (10-77 kDa) (Wang et al 2019), antiobesity agents (0.8 kDa) (Lin et al 2017), antidiabetic agents (5-30 kDa) (Wang et al 2019), and proangiogenic agents (15-20 kDa) (Marinval et al 2016). Various studies on the use of fucoidan have been carried out, especially as candidate drugs for both humans and animals. However, fucoidan has a molecule that is

too large as a drug candidate, so studying fucoidan with low molecular weight has its importance. Enzymatic hydrolysis can be used to study the structure of fucoidans and their oligomer production (Kusaykin et al 2016). The enzyme will work specifically on one type of bond in the polymer molecule and can be used for the depolymerization of fucoidan.

Fucoidanase is an enzyme that can degrade fucoidan, subsequently forming smaller molecules. This enzyme catalyzes the breaking of glycosidic bonds between fucose sulfate residues in fucoidan. This enzyme has been found in marine organisms such as bacteria (Silchenko et al 2013), invertebrates (Bilan et al 2005), and some fungi (Rodriguez-Jasso et al 2010). According to Rasin et al (2020), recombinant FFA1 fucoidanase from marine bacteria *Formosa algae* KMM 3553^T was able to degrade fucoidan from *Sargassum horneri* from an initial molecular weight of 140 kDa to 63 kDa. Zueva et al (2020) added that the fucoidanase fwf1, fwf2, fwf3, and fwf4 from the marine bacterium *Wenyingzhuangia fucanilytica* CZ1127^T can also hydrolyze the fucoidan from *Fucus evanescens* weighing 160 kDa to 88.78 kDa, 98.16 kDa, 106.91 kDa, and 83 kDa. Endo-α(1,3)-fucoidanase Mef2 from *Muricauda eckloniae* was also reported to be able to hydrolyze *Saccharina latissima* fucoidan from a molecular weight of 350 kDa to 200 kDa (Tran et al 2022).

On the other hand, *Sargassum* sp. was reported to contain symbiotic bacteria that can be used as candidates for fucoidan-degrading bacteria. The use of symbiotic bacteria on algae is more profitable than the use of algae because bacterial growth is easy to control, can be propagated in a short time, and the number of samples required is small (Subaryono 2019). So far, studies on the use of brown algae symbiont bacteria as fucoidan degraders have not been reported. Therefore, it is necessary to conduct further research on the use of brown algae symbiont bacteria as a candidate for fucoidanase-producing bacteria. This study aims to isolate, identify, and characterize fucoidanase-producing bacteria from brown algae morphologically, biochemically, and molecularly using the 16S rDNA PCR technique.

Material and Method

Sargassum polycystum collection. Samples of *Sargassum polycystum* brown seaweed were collected from Lampung waters, Sebalang Beach, Katibung District, South Lampung Regency in July 2022. The samples were placed in plastic containers and stored in a cool box to be transported to the Aquaculture Laboratory, Faculty of Agriculture, University of Lampung, for further processing.

Extraction of fucoidan from S. polycystum. Fucoidan extraction was carried out using the acid method (Setyawan et al 2018). 50 g of refined *S. polycystum* was weighed and macerated in 0.1 N HCl (1:10 m/v) (Merk) for 24 h. The algal sample was then macerated again in 0.2 N HCl (1:10 m/v) for 24 h. The extract was then filtered with a white cloth to separate the filtrate and precipitate. The two extracts were then combined and filtered using Whatman paper number 40. The extract was then evaporated using rotary evaporation (IKA RV 10, Germany) at 60°C to obtain 100 mL of the final extraction volume. The extract was precipitated with cold 96% ethanol and stored in the refrigerator for 2 h. The extract was centrifuged at 3000 rpm for 15 min. The precipitate was dissolved in distilled water at pH 2 with the addition of calcium chloride ($CaCl_2$ Merck) at a final concentration of 2 M and allowed to stand for 24 h at room temperature (30°C). The extract was then centrifuged again at 3000 rpm for 15 min. The supernatant was collected and precipitated with cold 96% ethanol for 24 h. Centrifugation was again carried out at 3000 rpm for 15 min to collect the fucoidan precipitate. To obtain fucoidan, supernatant was precipitated by ethanol. The crude fucoidan was then analyzed for total carbohydrates and sulfates and confirmed using a Fourier transform infrared spectrophotometer (FTIR) (Thermo Nicolet 380 FTIR, Germany). Finally, the obtained fucoidan was freeze-dried and stored in 4°C until use. The determination of total carbohydrate content was performed by the phenol-sulfuric acid method and sulfate content in fucoidan was determined by $BaCl_2$ – gelatin method (Sinurat & Fithriani 2018).

Isolation of S. polycystum symbiont bacteria. The method of isolating symbiotic bacteria from brown algae referred to previous studies (Subaryono 2019). 10 g of fresh brown seaweed were chopped and 1 g was put into a test tube containing 9 mL of sterile seawater to obtain the first dilution (10^{-1}). The first dilution was homogenized using a vortex and 1 ml was taken with a sterile pipette and put into a test tube containing 9 mL of sterile seawater and obtained a dilution of 10^{-2}. The dilution process continues until the dilution was 10^{-3}. The results of the dilution (100 µL) were further diluted and leveled using a sterile spreading rod in a petri dish containing nutrient agar medium. The medium was incubated at 37°C for 2x24 h. The bacteria that developed were purified by the scratch method. Bacterial colonies were separated with an inoculation needle based on differences in color and colony shape on nutrient agar medium in new Petri dishes. The pure isolate obtained was stored on agar slant media. The growing bacterial colonies were isolated on the same medium and incubated for 24-48 h at 30°C until several different pure isolates were obtained.

Isolation of *S. polycystum* symbiont bacteria was also carried out using the streak plate method (Kumar et al 2016). 10 g of fresh seaweed were cleaned with sterile seawater. The thallus was removed and cut into two parts with a sterile knife. The thallus was planted into the nutrient agar medium by attaching the cleaved part with little pressure. The samples were incubated in an incubator at 36°C for 24-48 h. The growing bacterial colonies were isolated and purified.

Qualitative and semi-quantitative test of fucoidanase. Qualitative and semi-quantitative determination of fucoidanase activity was carried out following Sawant et al (2015) with some modifications. A total of 100 µl of bacterial symbiont cultures that had been incubated for 24 h were dripped and flattened using a spreader on a Petri dish containing nutrient agar (NA) medium with the addition of fucoidan 1 mg mL^{-1} and incubated for 1 h to allow the bacteria could diffuse into the media. Paper disc (ø 6 mm) was placed on the agar surface and dripped with 10% cetyl pyridinium chloride ($C_{21}H_{38}ClN$) solution. The nutrient agar medium was incubated for 24 h at 36^oC. Positive symbiont isolates showed fucoidanase activity in the presence of a clear zone around the paper disc. The positive isolates were then tested for fucoidanase activity semi-quantitatively 3 times with the same method as the qualitative assay. Bacterial isolates were cultured on nutrient agar medium containing fucoidan 1 mg mL^{-1} and dripped with 10% cetyl pyridinium chloride solution then incubated for 24 hours at 36°C. After the incubation period, the clear zone around the paper disc was determined. The fucoidanase activity index of the microbial strains indicated by CPC methods was computed based on the following equation (Subaryono 2019):

$$\text{Fucoidanase activity} = \frac{(\phi \text{ clear zone} - \phi \text{ bacterial colony})}{\phi \text{ clear zone}} \times 100\%$$

Fucoidanase production. Several isolates with the largest fucoidanase activity index were selected for the production of crude fucoidanase extract, which was obtained following the method used by Manivasagan & Oh (2015), with several modifications. The isolated bacteria were inoculated in 10 mL of nutrient broth (NB) medium using an inoculation loop and incubated at 36°C for 24 h. 5 mL of culture results were inoculated into a specific medium consisting of 5 g wheat powder, 1 g *S. polycystum* powder, 0.5 g glucose, 0.05 g $NaNO_3$, 0.05 g $MgSO_4.7H_2O$, 1 g NaCl , and seawater with a final volume of 100 mL, which has been sterile. Incubation was then carried out on bacteria and specific media at a shaker speed of 150 rpm at 28°C for 7 days. Enzyme production was carried out by taking 5 mL of culture and placing it in a falcon tube for centrifugation. Centrifugation was carried out at 10000 rpm for 10 min. Harvesting was carried out at different time intervals, namely 24, 48, 72, and 96 h. The supernatant obtained was a crude enzyme extract that measured protein content and enzyme activity. Protein content was determined by the Bradford method using the protein assay reagent (Himedia, Mumbai, India) with bovine serum albumin as the standard (Trang et al 2022).

Quantitative fucoidanase activity test. The crude enzyme extract filtrate was tested for fucoidanase activity using the DNS (3,5-dinitrosalicylic acid) method (Manivasagan & Oh 2015). The determination of fucoidanase activity was carried out by measuring reducing sugar levels by preparing 3 groups of test tubes consisting of samples, controls, and blanks. The sample tube consisted of 0.9 mL of mixed solution (1% (w/v) of fucoidan from *S. polycystum* with 0.1 M citric acid-sodium citric buffer, pH 6.0) added to 0.1 mL of enzyme filtrate and incubated at 50°C for 10 min. The reaction was terminated by the addition of 1 mL DNS reagent. The sample tube was heated in boiling water at 100°C for 7 min. After the sample was cooled to room temperature, 3 mL of distilled water were added. The control tube was filled with 0.9 mL of mixed solution (1% fucoidan from *S. polycystum* and 0.1 M citrate buffer pH 6) plus 0.1 mL of enzyme filtrate without incubation. The control tube was immediately heated in boiling water at 100°C for 7 min and cooled to room temperature. 3 mL of distilled water were added. The blank consisted of 1 mL of 1% fucoidan solution and 1 mL of DNS preheated in boiling water at 100°C for 7 min. 3 mL of distilled water were added to the cold blank. Each test tube was repeated 3 times and the absorbance was measured with a spectrophotometer at a wavelength of 540 nm to determine the reduced sugar content. One unit (U) of fucoidanase activity is defined as the amount of enzyme that releases 1 μmol of fucose per minute under the assay conditions.

Identification of fucoidanase-producing bacteria. Bacterial identification was carried out on bacteria that had the largest fucoidanase index. Identification was done by observing morphological, biochemical and genetic features. The morphological characterization of bacterial colonies included color, shape, elevation, and the edge of the bacterial colony. Biochemical observations included Gram test, catalase test, and fermentative oxidative test. Genetic identification was carried out by 16S rRNA sequencing assay. DNA extraction was conducted using a DNA purification kit against bacterial isolates that were 24 h old. The primers used in the 16S rRNA amplification process were fragment 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGCTACCTTGTTACGACTT-3'). These two primers were chosen because they are general primers often used to determine fucoidanase-producing bacteria, so the chance of having a match with the DNA band of the type of bacteria tested was large. PCR was performed with the GeneAmp PCR System 9700 (Applied Biosystem, Foster City, CA, USA) with the following amplification conditions: initial denaturation at 95°C for 5 min, denaturation at 95°C for 30 sec, annealing at 55°C for 2 min, extension at 68°C for 1.5 min, and the last extension at 68°C for 10 min. The 16S rRNA sequence was compared with the multiple sequence data in the GenBank database using the BLAST algorithm and the CLUSTA W program. A phylogenetic tree was created from the program.

Statistical analysis. Data of semi-quantitative and quantitative fucoidanase activity tests were tabulated and analyzed numerically in Microsoft Excel and one-way analysis of variance (ANOVA) was applied at a 95% confidence level ($p<0.05$) using the SPSS 26.0 software. Subsequent testing was carried out using Duncan's test, if there were significant differences determined between isolates.

Results. 20 isolates from the brown algae *S. polycystum* from Sebalang Beach, Katibung District, East Lampung Regency, were the candidate isolates of fucoidanase-producing bacteria obtained. Different bacterial isolation methods from *S. polycystum* produced visually different results.

Characteristics of fucoidan from S. polycystum. The value of the total carbohydrate test for fucoidan from *S. polycystum* was 20.65%, indicating that there are other components in the sample other than carbohydrates. The sulfate content in the crude extract of fucoidan was 6.9%. The results of this test are important for determining the composition of fucoidan and its bioactivity. The fourier transformed infrared (FTIR) spectra of the fucoidan extract is presented in Figure 1.

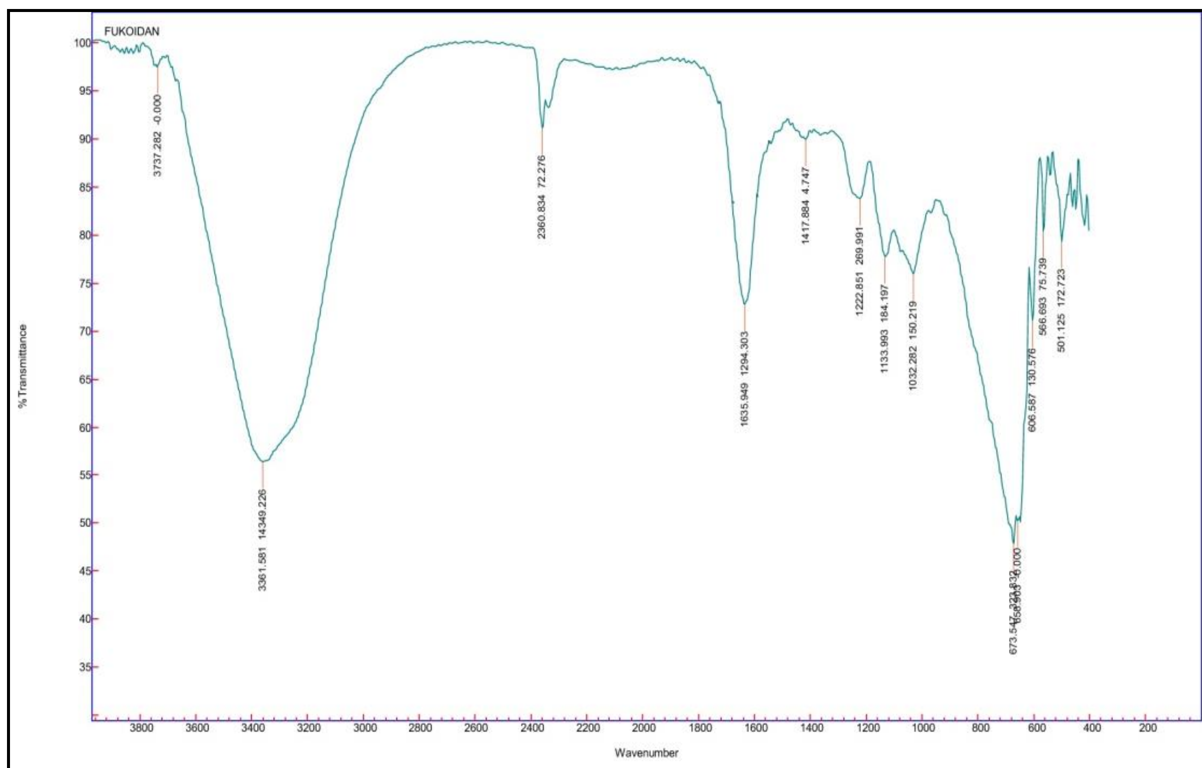

Figure 1. Fourier-transform infrared spectrum of *Sargassum polycystum* fucoidan.

Fucoidanase activity test. Based on the qualitative test of fucoidanase activity on 20 isolates of *S. polycystum* symbiont bacteria, it was found that 12 of them were positive for fucoidanase activity from the presence of a clear zone (Figure 2). The fucoidanase-active bacteria were studied by conducting a semi-quantitative fucoidanase activity test on the 12 isolates. The results showed that there were 4 isolates of symbionts positive for fucoidanase with a large index value, namely isolates of GSD, PTF, PSA, and GSB (Figure 3).

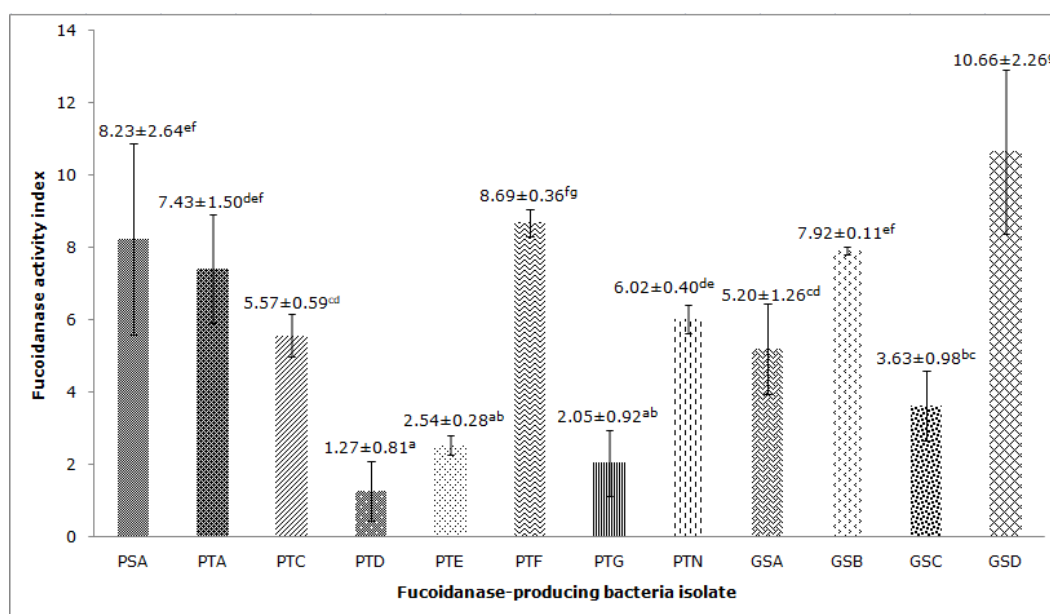

Figure 2. Fucoidanase activity index from 12 isolates of bacteria producing fucoidanase using the CPC method. Each value is the mean±SD of three replicates; bars with different letter are significantly different ($p<0.05$).

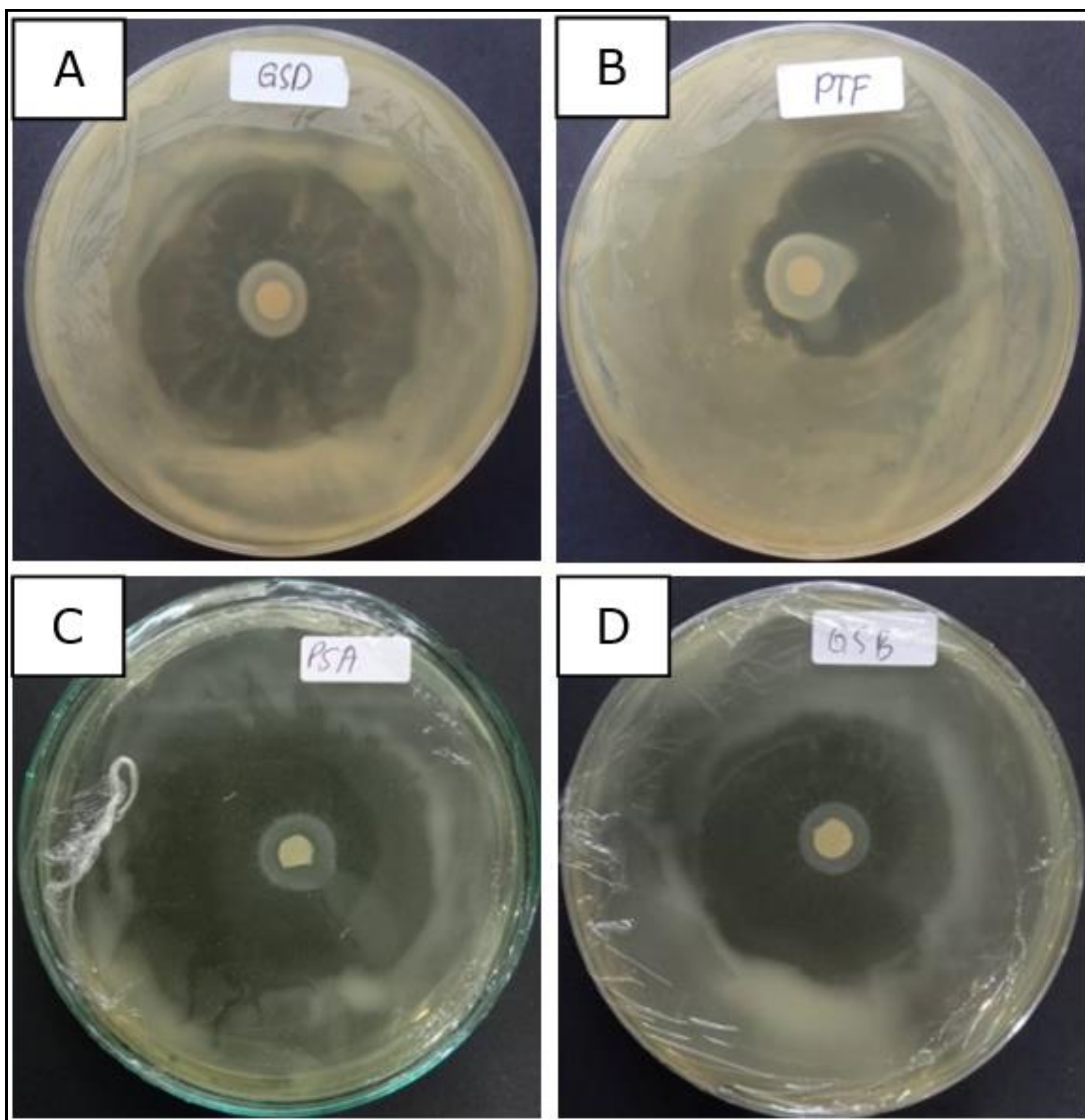

Figure 3. Qualitative and semi quantitative assay of detection of extracellular fucoidanase production by 4 bacterial isolates with the largest fucoidanase index: bacteria were grown for 24 h and paper disc dripped with CPC to display clear zones on fucoidan-agar plates. A - GSD isolate; B - PTF isolate; C - PSA isolate; D - GSB isolate.

The quantitative determination of fucoidanase activity against 4 isolates from *S. polycystum* symbionts found that the activity values were different. This can happen because each isolate is different. Based on observations on fucoidanase production after 24 h of incubation, PTF isolates had the highest activity of 0.0597 U mL^{-1}. Subsequent fucoidanase production after 48 and 72 h showed that GSD isolates had the greatest activity at 0.1475 U mL^{-1} and 0.1877 U mL^{-1}, respectively. The fucoidanase activity at harvest time of fucoidanase at 96 h showed a decrease in activity in all isolates (Figure 4).

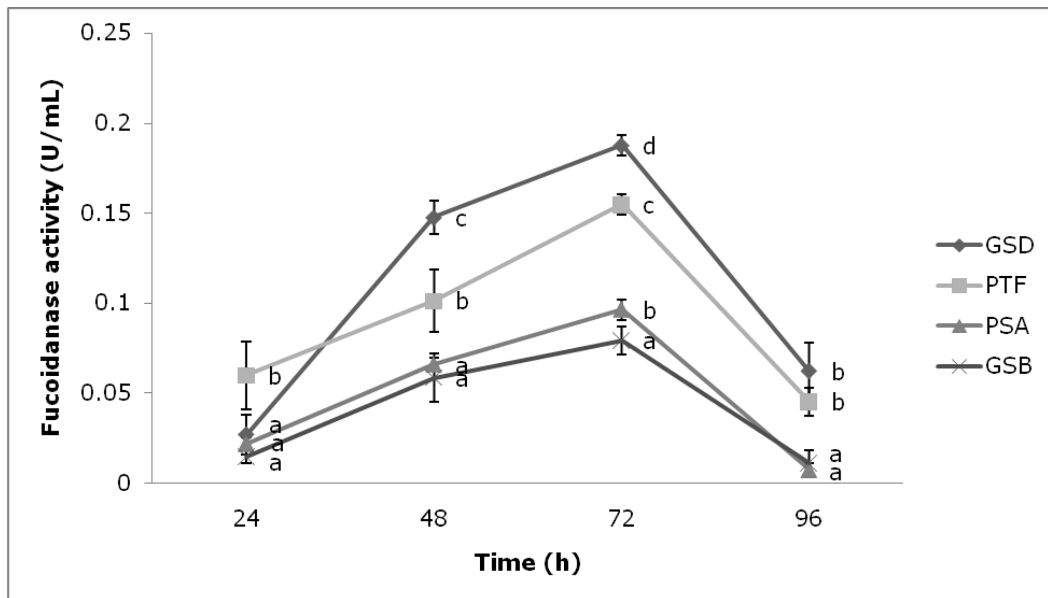

Figure 4. Quantitative fucoidanase activity against 4 isolates of the symbiotic bacterium *Sargassum polycystum* with the highest fucoidanase index. Each value is the mean±SD of three replicates; bars with different letters are significantly different ($p<0.05$).

The results of the calculation of fucoidanase protein levels at an incubation time of 24 to 72 h showed a significant increases in GSD, PTF, PSA, and GSB isolates. However, protein levels decreased after fucoidanase production was carried out at 96 h. Based on Figure 5, it can be seen that PTF isolates had the highest protein content at harvest time of fucoidanase at 24 to 72 h, which were 28.663 mg mL^{-1}, 58.580 mg mL^{-1}, and 66.136 mg mL^{-1}, respectively.

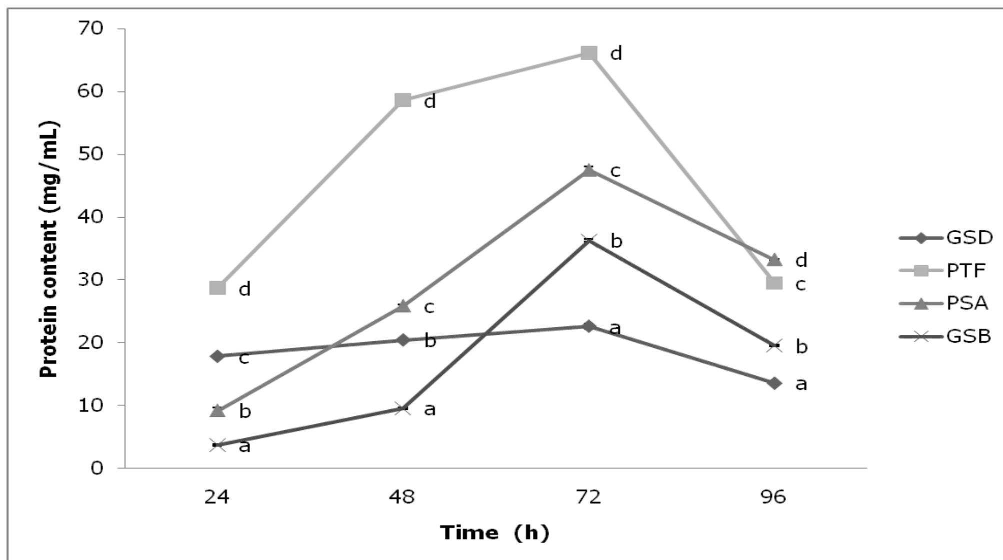

Figure 5. Fucoidanase protein levels in 4 isolates of *Sargassum polycystum* symbionts in specific media. Each value is the mean±SD of three replicates; bars with different letters are significantly different ($p<0.05$).

Based on the calculation of fucoidanase activity and protein content, the specific fucoidanase activity was calculated (Figure 6). The results showed that the fucoidanase production at 24 h of incubation of GSB isolates had the greatest activity of 0.0039 U mg^{-1}. The fucoidanase activity after 48 and 72 h showed that GSD isolates had the greatest activity compared to the other 3 isolates, namely 0.0072 U mg^{-1} and 0.0083 U

mg^{-1}. However, at harvest time of 96 h of incubation, it was seen that the four isolates had decreased fucoidanase activity.

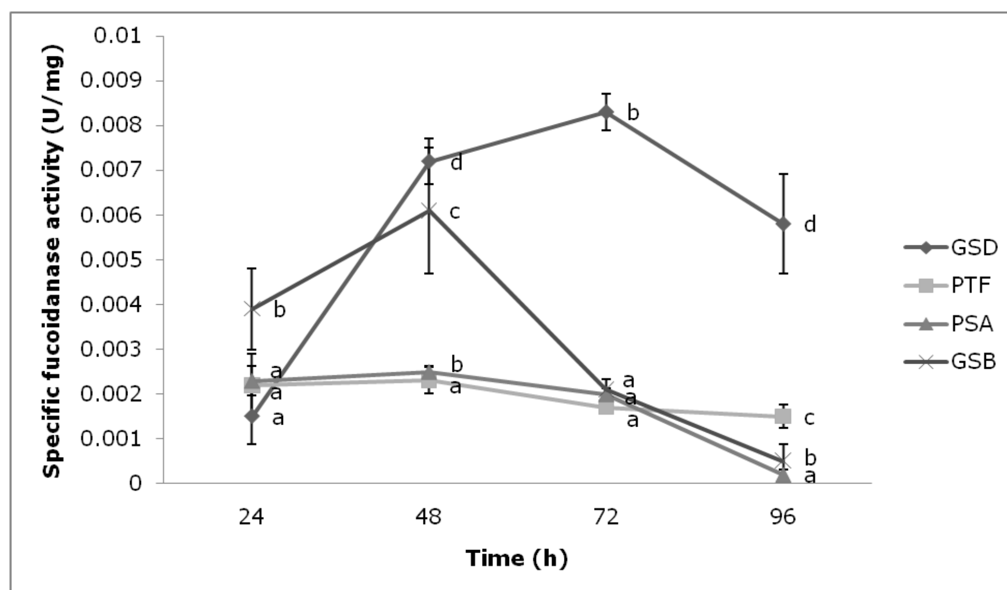

Figure 6. Specific fucoidanase activity of 4 isolates of *Sargassum polycystum* symbiont bacteria with the largest fucoidanase index. Each value is the mean±SD of three replicates; bars with different letters are significantly different ($p<0.05$).

Identification of fucoidanase-producing bacteria. Morphological and biochemical testing was carried out to support the molecular identification results from bacterial isolates. Morphological and biochemical results can be seen in Table 1.

Table 1
The results of morphological and biochemical observations of bacterial isolates with the greatest fucoidanase activity

Code of isolate	*Character*						
	Color	*Shape*	*Elevation*	*Edges*	*Gram*	*OF**	*Catalase*
GSD	Beige	Circular	Flat	Entire	+	Fermentative	+
PTF	Beige	Irregular	Convex	Undulate	+	Fermentative	+
PSA	Orange	Circular	Flat	Entire	+	Fermentative	+
GSB	Beige	Circular	Flat	Entire	+	Fermentative	+

Note: *OF - oxidative/fermentative reaction.

The visual characteristics of isolates of fucoidanase-producing bacteria showed that the four isolates had variations in color, shape, elevation, and edges. The 4 isolates, GSD, PTF, PSA, and GSB belong to the group of Gram-positive bacteria, characterized by the absence of mucus after administration of 3% KOH, is fermentative, and produces positive catalase.

Molecular identification using 16S rRNA PCR was carried out on 3 isolates, namely GSD, PTF, and PSA, while GSB was not identified because of its morphological and biochemical similarities with GSD. Sequence results and phylogenetic construction of the three isolates with the greatest fucoidanase activity led to 3 different bacterial species. The phylogenetic tree obtained from GSD showed similarities to *Cytobacillus kochii*, PTF had resemblance to *Bacillus cereus*, and PSA was similar to *Brevibacterium sediminis*. The results of the 16S rRNA PCR in the three isolates showed an identical value of 99% (Table 2). The phylogenetic tree of 3 symbiotic bacteria from *S. polycytum* with the greatest fucoidanase activity using the BLAST algorithm program and the CLUSTA W program is presented in Figure 7.

Table 2

Similarity percentage of nucleotide sequences from the 16S rRNA gene in bacterial isolates that produced the greatest fucoidanase activity

Code of isolate	*Bacteria name*	*Level of homology (100%)*	*Query/subject*	*Access number*
GSD	*Cytobacillus kochii* strain WCC 4582	99	1482/1488 (99%)	NR_117050.1
PTF	*Bacillus cereus* ATCC 14579	99	1517/1522 (99%)	AE016877.1
PSA	*Brevibacterium sediminis* strain CGMCC 1.15472	99	1467/1479 (99%)	KX356313.1

Discussion. The total carbohydrate content of fucoidan from *S. polycystum* in this study was lower than that from other brown algae. Several research results reported that the total carbohydrate content in brown algae is diverse, for example 43.26-46.82% in *Sargassum wightii*, 30.36-35.83% in *Dictyota dichotoma*, 34.81-35.47% in *Turbinaria decurrens*, 27.14-29.15% in *Padina boergesenii*, and 17.23-19.35% in *Stoechospermum marginatum* (Eluvakkal et al 2010), 63.45-72.40% in *Sargassum filipendula* (Sinurat & Fithriani 2018), and 62.90%-67.42% in *Padina* sp., *Sargassum* sp., and *Turbinaria* sp. (Lutfia et al 2020). The sulfate content of fucoidan from *S. polycystum* was large and similar to that discovered in previous studies. Fernando et al (2020) reported that the sulfate contained in *S. polycystum* from the south-west coast of Sri Lanka ranged from 12.42-33.56%. The amount of sulfate and the position of sulfate in the monosaccharide structure of fucoidan can affect its bioactivity. In addition, habitat and harvest season can also affect fucoidan bioactivity (Skriptsova et al 2009). Sulfate content in fucoidan is directly proportional to its ability as an antioxidant, with a greater percentage of sulfate rising its bioactivity as an antioxidant (Chen et al 2021). Differences in the composition of fucoidan in total carbohydrates and sulfate content can be caused by the living environment of the algae (temperature, ocean currents, growing depth, salinity), harvest time, species, growth stage, season, extraction process, type of extraction solvent, and time extraction (Skriptsova et al 2009; Sinurat et al 2016).

The FTIR spectrum of fucoidan from *S. polycystum* in this study showed a typical polysaccharide band and had a spectrum similar to fucoidan in other brown algae. There are 5 spectral bands in the sample, which strongly indicate that the sample is fucoidan, namely 3361.5 cm^{-1} (O-H), 1635.9 cm^{-1} (C=O), 1417.8 cm^{-1} (C-H), 1222.8 cm^{-1} (S=O), 1032.2 cm^{-1} (C-O). The strong and wide absorption peak at wave number 2750-3500 cm^{-1} represents the vibration of the O-H functional group (Isnansetyo et al 2016). The band in the wave number ranging from 1820-1600 cm^{-1} represents the C=O strain of the ester, which is thought to be the vibration of uronic acid (Marudhupandi et al 2014). The absorption band between 1470-1400 cm^{-1} represents the C-H group of fucose and an indication of the sulfate group bound to C_2 and C_4 of fucose, indicating the vibration of CH_2 (galactose and mannose) (Ale et al 2011). According to Li et al (2008), the absorption band at wave number 1200-1050 cm^{-1} shows CH vibrations of fucose and is an indication of fucose sulfate (S=O). Lutfia et al (2020) added that the absorption band at wave numbers between 1129.40-957.90 cm^{-1} is a characteristic of fucose and an indication of the stretching vibration of the S=O bound to the axial position of C-4. The characteristics of fucoidan are seen from the FTIR spectrum with absorption at a wave number of around 1200-800 cm^{-1} (Setyawan et al 2018).

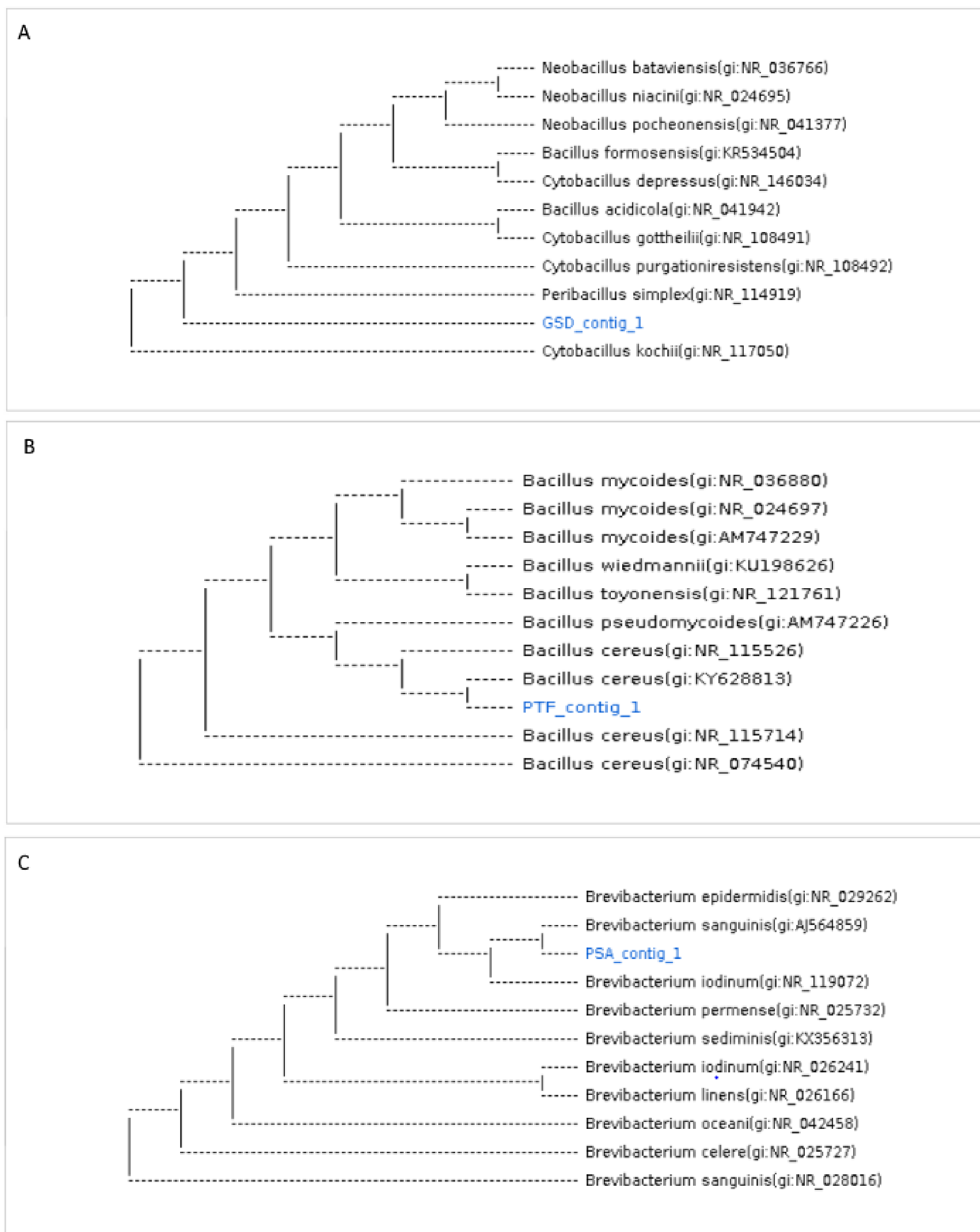

Figure 7. Phylogenic tree based on the nucleotide sequence of the 16S rRNA gene of symbiotic bacterial isolates that produced the greatest fucoidanase activity; A - GSD; B - PTF; C - PSA.

Qualitative and semi-quantitative enzyme testing on 20 candidate isolates of fucoidan-degrading bacteria from *S. polycystum* aimed to determine the presence of fucoidanase activity by looking at the clear zone. The presence of a clear zone indicated that the bacteria were able to degrade fucoidan in their growth media. The results of the isolation of bacterial symbionts on *S. polycystum* showed that 12 of the 20 isolates produced

fucoidanase activity. The range of fucoidanase activity index produced by the 12 isolates ranged from 1.27±0.81 to 10.66±2.13. 11 of the 12 isolates that were positive for fucoidanase had a high fucoidanase index value (fucoidanase index >2). The GSD isolate had the highest index and was significantly different from the other 10 isolates. 4 of the 12 isolates had the greatest fucoidanase activity: GSD, with a fucoidanase index of 10.66±2.26, PTF, with a fucoidanase index of 8.69±0.36, PSA, with a fucoidanase index of 8.23±2.64, and GSB, with a fucoidanase index of 7.92±0.11. The fucoidanase index can be used to predict the activity or amount of enzymes produced by a bacterium. The higher the index value, the higher the fucoidanase activity or the number of enzymes produced. This is following the research of Subaryono (2019), who used the same method to calculate the activity of alginate lyase-producing bacteria from *Sargassum crassifolium*.

Quantitative testing of fucoidanase activity showed that the 4 isolates had varied activities. Statistically, at harvest time of fucoidanase aged 24 h, PTF showed significant differences from the other 3 isolates ($p<0.05$), at harvest time fucoidanase aged 48 to 72 h, GSD had the greatest activity, significantly different from the other 3 isolates ($p<0.05$). The values obtained are lower when compared to previous studies. The study of Manivasagan & Oh (2015), using the same method, reported that the fucoidanase activity of *Streptomyces* sp. ranged from 2-16 U mL^{-1}. The research results of Qianqian et al (2011), using the same method, reported that the hydrolysis of fucoidanase using the marine fungus *Dendryphiella arenaria* resulted in a fucoidanase activity of 2.2 U mL^{-1}. The different lower yields can be caused by the different species of bacteria used to produce fucoidanase. In addition, the difference in the source of fucoidan as a substrate also greatly affects the activity produced.

The difference in the harvesting time of fucoidanase also affects its protein content. A good time for harvesting ranges from 48-72 h. The PTF isolate at harvest time of 48-72 h had the highest protein content and was significantly different from the other 3 isolates ($p<0.05$). The harvest time for fucoidanase at 24 h is also good, but the fucoidanase activity and protein content are not maximized. This is possible due to the condition of the bacteria that have not grown optimally. Meanwhile, the harvest time of fucoidanase at the age of 96 h of isolates is not recommended, due to a decrease in both the activity and protein content of fucoidanase. The decrease is thought to occur due to a large number of bacteria, causing competition and subsequently the death of some bacteria, reducing the total amount of enzyme production (Subaryono 2019).

Based on the calculation of the specific fucoidanase activity, it showed that the greatest activity at harvest time of 24 h occurred for GSB isolates, at 0.0039 U mg^{-1}, being significantly different from the other 3 isolates ($p<0.05$). The next harvest time continued to increase until the harvest time at 72 h. GSD showed the greatest fucoidanase activity at harvest times of 48 and 72 h, namely 0.0072 U mg^{-1} and 0.0083 U mg^{-1}, respectively. The results of the statistical analysis also showed that the specific fucoidanase activity of GSD isolates at harvest time was significantly different from that of the other 3 isolates ($p<0.05$). The binding between the enzyme and the substrate and its catalytic reaction depends on the interaction between the substrate and the amino acid side chains that make up the active site of the enzyme. This event occurs at the right ionization conditions for binding, and it is strongly influenced by the pH of the medium. All enzymatic reactions are affected by the pH of the medium in which the reaction occurs, and each enzyme has a unique optimum pH. At a certain pH, enzymes can lose their biological activity (Subaryono 2019).

The results of visual morphological observations on the four bacterial isolates showed that 3 isolates had a creamy color and 1 was orange. The colony forms of the 4 isolates were circular and irregular, the elevations were from flat to convex, and the edges were whole and undulated (Table 1). The results of this morphological test are in accordance with the research of Nurseha et al (2018), who reported that, generally, the color of colonies of bacteria in symbiosis with *S. polycystum* is creamy with a dominant convex and flat elevation. Sugrani et al (2019) added that bacteria that are symbiotic with the alga *Eucheuma spinosum* have almost the same colony morphological

characteristics, namely creamy or orange in color, circular and irregular in shape, flat and convex elevation with intact and wavy edges.

The results of the Gram reaction test on the 4 largest fucoidanase-producing bacterial isolates showed that none of the isolates produced mucus when testing the Gram reaction using 3% KOH. Thus, the 4 isolates with the highest fucoidanase values were classified as Gram-positive bacteria. Dash & Payyappilli (2016) added that there is no mucus formation because the cell walls of Gram-positive bacteria have a thick layer of peptidoglycan preventing it. The cell wall of Gram-positive bacteria has a layer of peptidoglycan (up to 90%), while Gram-negative bacteria have a high lipid content in their cell walls, with liposaccharides and lipoproteins (Dash & Payyappilli 2016). The O/F test showed fermentative properties of the 4 selected isolates. The 4 bacterial isolates were able to perform respiration using sugar under anaerobic or aerobic conditions. According to Mulyadi et al (2021), the color change in both O/F media to yellow indicates that the bacterial isolates can carry out respiration by utilizing glucose in the presence of oxygen (the tube is not covered with paraffin) and without oxygen (the tube is closed with paraffin). The catalase test on the 4 isolates showed positive catalase results, indicated by the formation of bubbles after being dripped with 3% H_2O_2 solution. This indicated that the 4 isolates produced catalase enzymes capable of hydrolyzing hydrogen peroxide (H_2O_2) into water (H_2O) and gas bubbles (O_2) (Stoica 2016).

Based on genetic identification with a 16S rRNA sequencing test, it was found that GSD isolates were most similar to *C. kochii*, PTF to *B. cereus*, and PSA to *B. sediminis*. In general, the identification results with 16S rDNA sequencing have a higher similarity when compared to biochemistry. This is probably due to the biochemical method (phenotypic), where more factors affect the identification results, such as the condition of the isolate, the age of the isolate, environmental conditions, and other factors that interfere with the metabolic ability of the bacteria (Subaryono 2019). The presence of *Cytobacillus kochii, Bacillus cereus*, and *Brevibacterium sediminis* bacteria as symbionts in *S. polycystum* is thought to be due to the availability of nutrients in *Sargassum* and a suitable environment for bacteria. Several previous studies also revealed that these 3 isolates are commonly attached to seaweed and marine sediments (Chen et al 2016; Subaryono 2019; Patel & Gupta 2020). Waters rich in salts do not prevent these bacteria from growing considering their ability to survive in an environment with a NaCl content of 0.5-25% (Sawant et al 2015; Patel & Gupta 2020; Rahman et al 2022).

Several species of bacteria can generally live and associate with seaweed due to the mutual symbiosis between the two. Seaweeds require certain vitamins, which are often supplied by the symbiotic bacteria. On the other hand, bacteria will utilize the cellular components of seaweed to produce certain enzymes (Subaryono 2019). Some of these bacterial species show host specificity because the components contained in one host will be different from those of other hosts (Villareal-Gomez et al 2010). Related to the results of this study, it is suspected that the fucoidan content in brown algae *S. polycystum* can be used as a carbon source by bacterial species from the *Bacillus* and *Brevibacterium* groups that have fucoidanase activity.

Conclusions. Based on the results of the study, it was concluded that there were 4 isolates of symbiotic bacteria from *S. polycystum* which had the greatest fucoidanase activity. The fucoidanase activity index values obtained were: for GSD isolate 10.66±2.26, for PTF isolate 8.69±0.36, for PSA isolate 8.23±2.64, and for the GSB isolate 7.92±0.11. The specific fucoidanase activity also showed that GSD isolates at 48 and 72 h of fucoidanase production had the greatest activity compared to the other 3 isolates, namely 0.0072 U mg^{-1} and 0.0083 U mg^{-1}, respectively. Morphological, biochemical, and molecular identification with 16S rRNA PCR found that the three isolates with the greatest fucoidanase activity were *Cytobacillus kochii* (GSD), *Bacillus cereus* (PTF), and *Brevibacterium sediminis* (PSA) bacteria, with 99% similarity.

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