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Research Article

Environmental Effect on the Growth and Enzyme Activity of Fucoidanase-Producing Bacteria *Cytobacillus kochii* GSD

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Abstract

Extensive research has shown that low molecular weight fucoidan exhibits significantly greater biological activity than its high molecular weight. *C. kochi* GSD, a Sargassum symbiont bacterium, is proven to have the activity of hydrolyze fucoidan. This study proposes the growth optimization and fucoidanase enzymatic activity of *C. kochii* GSD bacteria under varying environmental conditions (temperature, pH, and salinity) cultured in basic liquid medium (BLM) for 48 hours. Based on Response Surface Methodology (RSM), the range of temperature, pH, and salinity for the growth optimization test of *C. kochii* GSD bacteria were 26.591, 30, 35, 40, and 43.49oC, the pH used starts from 3.636, 5, 7, 9, and 10.363, while the salinity to be used starts from 3.522, 5, 17.5, 30, and 38.522 ppt, respectively. The best conditions for growth of each environment were then continued with the test of fucoidanase enzyme activity in vitro. The results showed that *C. kochii* GSD bacteria grew optimally at temperature, pH, and salinity of 35oC, 7, and 30 ppt, respectively. The optimum enzyme activity of *C. kochii* GSD is at 72 hours with the forming of clear zones on media containing fucoidan and given Cetylpyridinium chloride (CPC) solution with clear zone diameters of 31.17 mm (temperature), 25.9 mm (pH), and 20.97 mm (salinity), respectively. The conclusion of this study is a high novelty finding to produce low molecular weight fucoidan enzymatically with *C. kochii* GSD bacteria to be used as an immunostimulant.

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1. Introduction

Fucoidans are complex polysaccharides that comprise the cell membrane of brown algae (Phaeopyceae) such as *Sargassum polycystum*, *Sargassum henslowianum*, *Sargassum plagiophyllum*, *Sargassum silquosum*, *Turbinaria conoides*, *Sargassum crassifolium*, *Acaudina molpadioides* (Hu *et al*., 2017; Nagappan *et al*., 2017; Hikariastri *et al*., 2019; Puspantari *et al*., 2020; Sun *et al*., 2020; Panjaitan and Natalia, 2021; Saetan *et al*., 2021). The material of the fucoidan extract is affected by the extraction method, harvest age, species, weather, and environmental conditions (Sinurat and Kusumawati, 2017). Fucoidan has a variety of biological activities, which makes fucoidan an important research material, especially in medical and aquaculture fields (Suresh *et al*., 2013; Wang *et al*., 2019; Abdel-Latif *et al*., 2022). Fucoidan is useful for aquaculture as a supplement in fish feed (Abdel-Latif *et al*., 2022). The feed containing fucoidan can modulate the microbes in the gut of adult zebrafish, encourage the growth of barramundi (*Lates calcarifer*), and increase disease resistance in tiger shrimp (*Penaeus monodon*) (Tuller *et al*., 2012; Sivagnanavelmurugan *et al*., 2014; Ikeda-Ohtsubo *et al*., 2020).

Fucoidan has been studied to have various bioactivities such as anti-bacterial (Zhu *et al*., 2021), anti-proliferation of melanoma cells (Malyarenko *et al*., 2021), antioxidant (Cholaraj and Venkatachalam, 2024), triggering apoptosis of colon cancer cells in humans (Srimongkol *et al*., 2022), antitumor/cancer (Sung *et al*., 2022), antiviral (Chen and Li, 2023), and immunostimulant in fish (Vijayaram *et al*., 2022) and shrimp (Setyawan *et al*., 2018). Fucoidan has a high molecular weight, which often limits its biological activity. Therefore, a method is needed to break down fucoidan molecules into smaller sizes to enhance their effectiveness. Previous studies have demonstrated the efficacy of low molecular weight fucoidan. Low molecular weight fucoidan has better bioactivity than high molecular weight fucoidan. This was proven in several studies, such as 45 kDa (Fernando *et al*., 2021), 2.3 kDa (Wang *et al*., 2024), and 90.8 kDa (Sun *et al*., 2023). Various methods can produce low-molecular fucoidan such as enzymatic hydrolysis (Kusaykin *et al*., 2016), free radical degradation, ultrasonic degradation, and high-temperature degradation (Wang *et al*., 2021). Enzymatic hydrolysis is the most effective method because it is easier, more efficient, and controllable. Enzymatic hydrolysis studies fucoidan structure and oligomer production (Ma *et al*., 2024). The enzyme will act specifically on one particular bond type in the polymer molecule and is used to depolymerize fucoidan (Kusaykin *et al*., 2016).

However, all earlier studies primarily focused on Fucoidanase. It is an enzyme that breaks down fucoidan, reducing its molecular weight. Fucoidanase is an enzyme that can degrade fucoidan so that the molecular weight is lowered (Abbas *et al*., 2025). This enzyme has been found in marine organisms such as bacteria (Silchenko *et al*., 2018), invertebrates (Pavão and De Souza Cardoso, 2022), and some fungi (Fahmid *et al*., 2023). According to research by Rasin *et al*. (2020), recombinant fucoidanase FFA1 from the marine bacterium *Formosa* algae KMM 3553T was able to degrade *Sargassum horneri* fucoidan from an initial molecular weight of 140 kDa to 63 kDa. Zueva *et al*. (2020) added that fucoidanases fwf1, fwf2, fwf3, and fwf4 from marine bacteria *Wenyingzhuangia fucanilytica* CZ1127T can also hydrolyze *Fucus evanescens* fucoidan with a weight of 160 kDa to 88.78 kDa, 98.16 kDa, 106.91 kDa, and 88.83 kDa. Endo-α (1,3)-fucoidanase Mef2 produced by the marine bacterium *Muricauda eckloniae* was also reported to hydrolyze *Saccharina latissima* fucoidan from a molecular weight of 350 kDa to 200 kDa (Tran *et al*., 2022). Very few studies have been conducted concerning the observed growth performance of *Sargassum* symbiont bacteria from Indonesian waters.

A previous study has found three isolates of *Sargassum* symbiont bacteria from Lampung Waters, namely *Cytobacillus kochii* GSD, *Bacillus cereus* PTF, and *Brevibacterium sediminis* PSA, that have fucoidanase activity in vitro (Setyawan *et al*., 2023). Low molecular weight fucoidan (LMWF) synthesis can be carried out enzymatically mediated by the fucoidanase enzyme. Therefore, the discovery of fucoidanase-producing bacteria, *C. kochi* GSD, plays an important role in LMWF, which is expected to be an immunostimulant in aquaculture sectors. However, further research is needed to determine the optimization of bacterial growth, bacterial pathogenicity, optimization of fucoidanase activity, and enzymatically low molecular weight of fucoidan (ELMW Fucoidan) for immunostimulants in vaname shrimp and grouper fish, which are one of the main aquaculture commodities in Indonesia. This study has a high novelty level related to fucoidanase-producing bacteria. Low molecular weight fucoidan is expected to be applied in various fields, especially as an immunostimulant for shrimp and grouper cultures. The results of this study are expected to optimize bacterial growth by environmental and media manipulation and enzyme activity of the fucoidanase-producing bacteria *C. kochii* GSD.

2. Materials and Methods

2.1 Materials

2.1.1 The equipments

The equipment in this study is a UV-Vis Spectrophotometer (Thermo fisher scientific, United States, 2023), pH meter (Schott instrument, UK, 2023), autoclave (Hirayama HVE-50, Japan, 2023), laminar airflow (Kojair, Finland, 2020), vortex (Maxi Max II-Thermo Scientific, China, 2024), water bath shaker (Thermo Scientific, China, 2024), measuring cup (Pyrex, United States, 2024), Erlenmeyer (Pyrex, United States, 2024), test tube (Pyrex, United States, 2024), thermometer, micropipette (Thermo fisher scientific, United States, 2023), dropper pipette, micro tube, cool box, magnetic stirrer, hot plate, analytical balance, bunsen, tweezers, cotton, aluminum foil.

2.1.2 The materials

The materials used in the study are *S. polycystum*, bacterial isolates of *C. kochii* GSD that have been screened and characterized morphologically, biochemically, and molecularly in previous studies which were isolated from brown algae *S. polycystum* in South Lampung waters, K2HPO4, (NH4)2SO4, MgSO4.7H2O, NaCl, Yeast extract agar, Yeast extract powder, NA agar, NA broth, *Bacillus cereus* Hiveg agar base (HIMEDIA), Zobell marine agar (Himedia), Zobell marine broth (Himedia), CaCl2.2H2O, distilled water, sodium citrate buffer, NaOH, Cetyl Pyridinium Chloride (CDH), dinitro salicylic acid reagent (DNS).

2.1.3 Ethical approval

This study does not require ethical approval because it does not use experimental animals.

2.2 Methods

The research was conducted at the Laboratory of Aquaculture, Oceanography, Biotechnology, and Agricultural Product Technology, Faculty of Agriculture, University of Lampung.

2.2.1 Experimental design

This study was designed using the Response Surface Methodology (RSM). The Response Surface Methodology (RSM) model is used to see the optimal conditions of temperature, pH, and salinity on the growth of *C. kochii* GSD bacteria in producing fucoidanase enzyme from *S. polycystum*. The independent variables/x factors are temperature, pH, and salinity, which are used to analyze the y response (fucoidanase enzyme activity). The experimental design used was a three-factor Central Composite Design (CCD). From the CCD results, a value of 1.681 was obtained. The center point of the temperature variation in this study refers to the results of stability tests from work by Li *et al*. (2021); the optimum temperature of fucoidanase crude extract activity is 20-50°C. Determination of the optimum pH of fucoidanase enzyme activity also refers to the findings of Li *et al*. (2021), who state that the optimum pH of fucoidanase enzyme activity is at pH 4. Meanwhile, the optimum salinity determination is obtained from Response Surface Methodology (RSM), which is 3.5 - 38 ppt.

From the designs using Response Surface Methodology (RSM), it is observed that the temperature parameters to be used start from 26.591, 30, 35, 40, and 43.409°C, then the pH used starts from 3.636, 5, 7, 9, and 10.363, while the salinity to be used starts from 3.522, 5, 17.5, 30, and 38.522 ppt. The total experimental units tested based on the CCD can be seen in Table 1. The outcomes of the design using CCD continued to be an experiment that obtained as many as 20 experimental units. The data were analyzed using Minitab Express to map the response surface and identify the optimal experimental conditions.

2.2.2 Procedure for sample preparation

The best isolated sample of *C. kochii* GSD bacteria was found from the results of a specific fucoidanase enzyme confirmation test from previous research conducted by Setyawan *et al*. (2023). Although the ability of these bacterial isolates to produce fucoidanase enzymes has passed the qualitative testing stage, namely by measuring the clear zone, the steps in measuring the clear zone begin with using a Petri dish containing agar media that has been inoculated with *C. kochii* GSD bacteria. After that, incubate for 48 hours and observe the clear zone. For quantitative measurement, use a ruler or vernier caliper to measure the diameter of the clear zone (in mm) vertically, horizontally, and diagonally, then take the average of the diameter or clear zone (Pazla *et al*., 2024). Further quantitative testing is needed to determine their ability to produce fucoidanase enzymes.

2.2.3 Preparation of medium

The basic liquid medium used is Nutrient Agar (NA) and Nutrient Broth (NB). The following table shows the quantity of reagents required for the preparation of nutrient agar in 100 ml and 1000 ml (Table 2). The mixed liquid medium with pH 6.8-7 distilled water was then sterilized using an autoclave at 121°C for 15 minutes and cooled to 50°C. NA and NB medium were kept in the fridge 4°C with sealed of sterile tube until 24 hours to prevent contamination or degradation.

2.2.4 Effect of temperature, pH, and salinity

Five Erlenmeyer flasks, each containing 100

Table 1. Optimization of temperature, pH, and salinity on fucoidanase enzyme activity using RSM with CCD method.

No	Code			Treatment		
	X1	X2	X3	Temperature (°C)	pH	Salinity (ppt)
1	-1	-1	-1	30	5	5
2	1	-1	-1	40	5	5
3	-1	1	-1	30	9	5
4	1	1	-1	40	9	5
5	-1	-1	1	30	5	30
6	1	-1	1	40	5	30
7	-1	1	1	30	9	30
8	1	1	1	40	9	30
9	-168.179	0	0	26.591	7	17.5
10	168.179	0	0	43.409	7	17.5
11	0	-168.179	0	35	3.636	17.5
12	0	168.179	0	35	10.363	17.5
13	0	0	-168.179	35	7	3.522
14	0	0	168.179	35	7	38.522
15	0	0	0	35	7	17.5
16	0	0	0	35	7	17.5
17	0	0	0	35	7	17.5
18	0	0	0	35	7	17.5
19	0	0	0	35	7	17.5
20	0	0	0	35	7	17.5

Table 2. Preparation of nutrient agar and nutrient broth medium.

Medium	Component	Quantity in grams for 1000 ml (g)	Quantity in grams for 100 ml (g)
Nutrient Agar	Beef extract	3	0.3
	Peptone	5	0.5
	Sodium chloride	5	0.5
	Agar	20	2
	Distilled water	1000 ml	100 ml
Nutrient Broth	Beef extract	3	0.3
	Peptone	5	0.5
	Sodium chloride	5	0.5
	Distilled water	1000 ml	100 ml

mL of Nutrient Agar (NA) and Nutrient Broth (NB) in triplicate, resulted in 15 experimental units. These were inoculated with *C. kochii* GSD isolates and incubated for 48 hours at various temperatures, such as 26,591, 30, 35, 40, and 43,409ºC, several pHs, such as 3.636, 5, 7, 9, and 10.363, and diverse salinities, such as 3.522, 5, 17.5, 30, and 38.522 ppt, to observe the optimum growth.

2.2.5 Measurement of bacterial growth and preparation

Preparing bacterial cultures is an important first step before calculating the density of bacteria. Nutrient Broth (NB) media is used to prepare the culture. The first step is inoculation, which involves adding bacteria to sterile liquid media in an Erlenmeyer flask. The culture was then incubated in a shaker incubator at 150-200 rpm at 37°C for 16-24 hours until the media looked turbid as an indicator of bacterial growth. Once the bacterial culture was ready, the density of bacteria was done by two methods, specifically spectrophotometry (OD620), which measures the turbidity of the bacterial solution at a wavelength of 620 nm using a spectrophotometer and observed every 3 hours throughout 24 hours. The bacteria were calculated using the linear regression formula y = a + bx. Where y = dependent variable, x = independent variable, a = constant, and b = regression coefficient. In addition, culture bacteria were also calculated using the colony count method (Colony Forming Unit, CFU/mL). In this method, serial dilution was carried out by diluting the bacterial culture gradually, then 100 µL of each dilution was grown on agar medium and incubated for 24 hours. After that, the number of colonies that grew was counted and used to calculate the concentration of bacteria in the initial sample using the CFU/mL formula.

2.2.6 Fucoidanase enzyme activity assay

The crude enzyme extract filtrate was analyzed for fucoidanase activity using the DNS (3,5-dinitrosalicylic acid) method (Manivasagan and Oh, 2015). Fucoidanase activity was measured by determining the reduction in sugar levels using three groups of test tubes: samples, controls, and blanks. The sample tube consisted of 0.9 ml of mixed solution (1% *S. polycystum* fucoidan and 0.1 M citrate buffer pH 6) added with 0.1 ml of enzyme filtrate and then incubated at 50ºC for 10 minutes. The reaction was then stopped by adding 1 ml of DNS reagent. The DNS reagent was made based on Subaryono's studies (2019). The sample tube was heated in boiling water at 100ºC for 7 minutes. After the sample is cooled to room temperature, 3 ml of distilled water is added.

The control tube was filled with 0.9 ml of mixed solution (1% *S. polycystum* fucoidan 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 100ºC for 7 minutes. The mixture was then cooled to room temperature, and 3 mL of distilled water was added. The blank consisted of 1 ml of 1% fucoidan solution and 1 ml of DNS, first heated in boiling water at 100ºC for 7 minutes. The blank that has been cooled then added 3 ml of distilled water. Each test tube was repeated 3 times, and then the absorbance was measured with a spectrophotometer at a wavelength of 540 nm to determine the reduced sugar content. One unit of enzyme is expressed as the amount of enzyme that releases one µmol of reducing sugar per minute. The fucose concentration was converted to IU/mL: IU/mL = 1 µmol/min of glucose produced = 0.18 mg/min of glucose. The activity of the crude enzyme extract of fucoidanase was calculated with the following equation (Murtiyaningsih and Hazmi, 2017):

Fucoidanase Activity (U/ml) = (Produk Concentration x df x 10) / (Incubation time x MW fukosa).....(i)

Where :

df : dilution factor

MW fucose: molecular weight of fucose

2.3 Analysis Data

The data collected in this research are qualitative and quantitative. The qualitative data obtained were presented descriptively. Furthermore, quantitative data from the growth curve of *C. kochii* GSD bacterial isolate was plotted against incubation time. The data from optimizing fucoidanase enzyme activity concerning temperature, pH, and salinity are presented in graphical form and interpreted based on the results.

3. Results and Discussion

3.1 Results

3.1.1 The physical characteristics of raw materials

The physical characteristics of fucoidan yield from *S. polycystum* are presented in Table 3. The extraction process of fucoidan from *S. polycystum* yielded 1.19% ± 0.29, with variation between repetitions ranging from 1.30% to 1.40% (Table 3). This relatively low yield is due to the stepwise extraction method, which involves adding calcium chloride (CaCl2) and ethanol.

Table 3. Fucoidan yield (%) from *S. polycystum*.

Dry Weight *S. polycystum* (g)	Wet Weight Fucoidan (g)	Dry Weight Fucoidan (g)	Fucoidan yield (%)
50	1.67	0.59	3.34

3.1.2 Effect of temperature

The effect of temperature on the growth of *C. kochii* GSD (Figure 1a). Figure 1a shows the temperature differences, including 26.5°C, 30°C, 35°C, 40°C, and 43.4°C. The growth data are presented in Table 4. The effect of varying temperatures on the growth of *C. kochii* GSD bacteria is reflected in their growth rate, the number of generations, and bacterial generation time.

The growth of *C. kochii* GSD in Figure 1a shows the absolute value with each polynomial at each temperature. The best growth was recorded at the peak of the polynomial at 35°C with $y = -0.0162x^2 + 0.5228x$ $R^2 = 0.5169$ and 40°C with $y = -0.0177x2 + 0.543x$ $R^2 = 0.1519$. In addition, the lowest value was found at 26.5°C with $y = -6E+06x2 + 2E+08x$ $R^2 = 0.0234$. Two different temperatures have the highest growth value, which shows the variation of optimal conditions for *C. kochii* GSD to achieve maximum cell density. However, 35°C was the optimum temperature for the growth of *C. kochii* GSD in preparation for the subsequent experiment on the effect of pH.

3.1.3 Effect of pH

The changing pH conditions might be another factor affecting the growth effectiveness of *C.* GSD bacteria. pH values in this study from 3.6, 5, 7, 9, and 10.3 are good choices for representing acidic, neutral, and alkaline conditions. Figure 1b presents the effect of pH on the growth of *C. kochii* GSD bacteria; the results illustrate that all pH values show different growth activities. pH 7 shows the highest cell density with a polynomial peak value of $y = -0.0151x^2 + 0.4851x$ $R^2 = 0.681$. Bacteria growth at pH 7 is quite high and constant, indicating that this condition is the most optimal for bacterial growth and the experiment on the effect of salinity.

3.1.4 Effect of salinity

The effect of salinity concentration on 3.5, 5, 17.5, 30, and 38.5 ppt on the growth of *C. kochii* GSD bacteria (Figure 1c). The results showed that salinity optimization affected the development of *C. kochii* GSD bacteria. Salinity optimization with 30 ppt is the optimum condition for the polynomial of $y = 0.0329x + 2.9187$, $R^2 = 0.5148$. The highest bacterial density at 30 ppt salinity was at the 21st hour. Therefore, 30 ppt salinity was confirmed as suitable for the growth of *C. kochii* GSD bacteria. In summary, temperature, pH, and salinity all influence bacterial growth. Finally, the fucoidanase enzyme activity assay was conducted using the optimal conditions of 35°C for 24 hours, a pH of 7, and a salinity of 30 ppt.

3.1.4 Fucoidanase enzyme activity assay

The results of the fucoidanase enzyme activity of *C. kochii* GSD during the 96-hour observation period parameters (Table 5). The hydrolysis process was monitored under optimal environmental conditions to maximize the production of fucoidanase.

During a 96-hour observation period, the highest activity was observed at 72 hours, as evidenced by clear zones measuring 31.17 mm (temperature) (Figure 2a), 25.9 mm (pH) (Figure 2b), and 20.97 mm (salinity) (Figure 2c). Cetylpyridinium chloride (CPC) reacted with fucoidan to produce a clear zone.

3.2 Discussion

3.2.1 Effect of environmental test

Environmental testing analysis revealed varied polynomial bacterial growth patterns across five media under different temperatures, pH levels, and salinity conditions. The growth of *C. kochii* GSD on the media was measured using a spectrophotometer with a wavelength of 620 nm. The wavelength range commonly used in antibacterial tests using a spectrophotometer is 600-620 nm because in that wavelength range, the turbidity of the solution can appear, which varies from yellow to brown (Furqonita *et al.*, 2021; Tzevelekidis *et al.*, 2024). Based on the data in Table 4, bacterial growth at various temperatures ranging from 26.5°C, 30°C, 35°C, 40°C, to 43.4°C showed diverse characteristics during the observation time (0 to 24 hours). At 26.5°C, bacterial density increased until the 15th hour with a density of 1.73×10^9 CFU/ml but decreased to 1.75×10^9 CFU/ml at the 24th hour. It is confirmed by Wang *et al.* (2024) stated that the lower temperature of 16°C caused a sudden decrease in ni

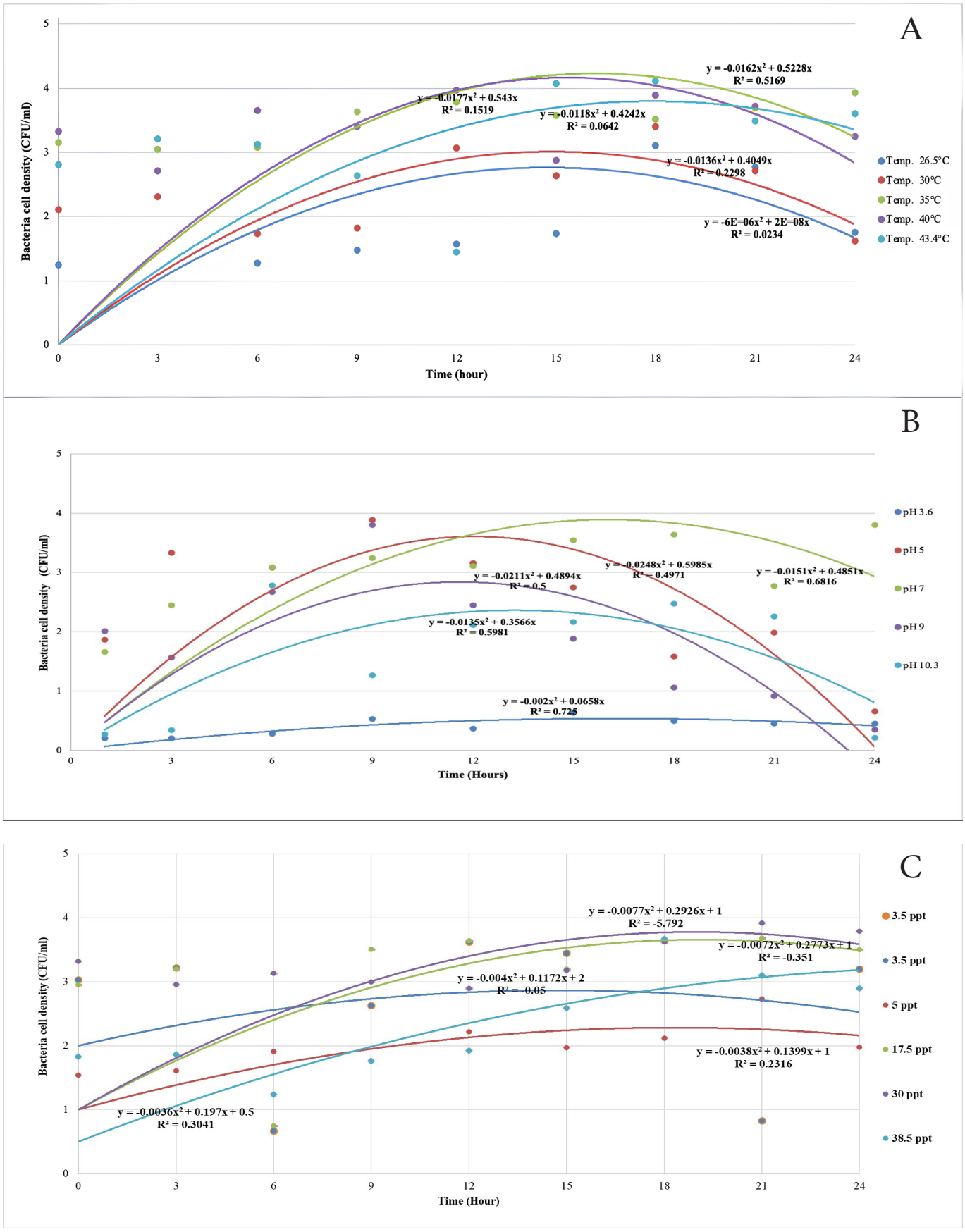

Figure 1. The results of the environmental effect in (a) temperature, (b) pH, (c), and salinity on growth *C. kochii* GSD.

Table 4. Growth data on *C. kochii* GSD in environmental effect test (Temperature, pH, and salinity).

Parameter			Observation Time (Hour)								
			0	3	6	9	12	15	18	21	24
Bacterial Density (CFU/ml)	Temp (°C)	26.5	1.24×10^{9}	1.23×10^{9}	1.27×10^{9}	1.47×10^{9}	1.57×10^{9}	1.73×10^{9}	3.1×10^{9}	2.77×10^{9}	1.75×10^{9}
		30	2.11×10^{9}	2.31×10^{9}	1.73×10^{9}	1.82×10^{9}	3.70×10^{9}	2.63×10^{9}	3.4×10^{9}	2.71×10^{9}	1.62×10^{9}
		35	3.15×10^{9}	3.05×10^{9}	3.08×10^{9}	3.63×10^{9}	3.78×10^{9}	3.57×10^{9}	3.52×10^{9}	3.69×10^{9}	3.93×10^{9}
		40	3.32×10^{9}	2.71×10^{9}	3.65×10^{9}	3.4×10^{9}	3.9×10^{9}	2.87×10^{9}	3.89×10^{9}	3.72×10^{9}	3.25×10^{9}
		43.4	2.81×10^{9}	3.21×10^{9}	3.12×10^{9}	2.63×10^{9}	1.45×10^{9}	4.07×10^{9}	$4.11\times10\times$	3.49×10^{9}	3.60×10^{9}
	pH	3.6	0.19×10^{8}	0.19×10^{8}	0.27×10^{8}	0.52×10^{8}	0.36×10^{8}	0.62×10^{8}	0.49×10^{8}	0.48×10^{8}	0.44×10^{8}
		5	1.86×10^{9}	3.33×10^{9}	3.08×10^{9}	3.89×10^{9}	3.16×10^{9}	2.75×10^{9}	1.58×10^{9}	1.98×10^{9}	0.658×10^{9}
		7	1.66×10^{9}	2.45×10^{9}	3.09×10^{9}	3.24×10^{9}	3.11×10^{9}	3.54×10^{9}	3.64×10^{9}	2.77×10^{9}	3.8×10^{9}
		9	2.01×10^{9}	1.56×10^{9}	2.67×10^{9}	3.80×10^{9}	2.45×10^{9}	1.88×10^{9}	1.06×10^{9}	0.91×10^{9}	0.348×10^{9}
		10.3	0.27×10^{8}	0.34×10^{8}	2.78×10^{8}	1.26×10^{8}	2.11×10^{8}	2.16×10^{8}	2.47×10^{8}	2.26×10^{8}	0.21×10^{8}
	Salinity (ppt)	3.5	3.03×10^{9}	3.22×10^{9}	0.66×10^{9}	2.63×10^{9}	3.62×10^{9}	3.45×10^{9}	3.64×10^{9}	0.83×10^{9}	3.2×10^{9}
		5	1.54×10^{9}	1.61×10^{9}	1.91×10^{9}	1.76×10^{9}	2.22×10^{9}	1.97×10^{9}	2.12×10^{9}	2.73×10^{9}	1.98×10^{9}
		17.5	2.95×10^{9}	3.2×10^{9}	0.75×10^{9}	3.51×10^{9}	3.64×10^{9}	3.2×10^{9}	3.63×10^{9}	3.68×10^{9}	3.5×10^{9}
		30	3.32×10^{9}	2.96×10^{9}	3.13×10^{9}	3×10^{9}	2.9×10^{9}	3.18×10^{9}	3.62×10^{9}	3.92×10^{9}	3.79×10^{9}
		38.5	1.83×10^{9}	1.86×10^{9}	1.24×10^{9}	1.76×10^{9}	1.92×10^{9}	2.59×10^{9}	3.67×10^{9}	3.1×10^{9}	2.9×10^{9}

Table 5. Clear zone values during the 96-hour. observation period.

Parameters	Clear Zone (mm)			
	24 h	48 h	72 h	96 h
Temperature (35°C)	17.17	28.63	31.17	30.85
pH (7)	24.5	24.8	25.9	22.7
Salinity (30 ppt)	11.72	19.33	20.97	20.23

trogen loss and viability. Lower temperatures (<25°C) can reduce the performance of microorganisms in the anaerobic digestion process. Therefore, 26.5°C is not suitable for the optimal growth of *C. kochii* GSD bacteria. At 30°C, bacterial growth was visibly increased compared to 26.5°C, with a density of 3.70×10^{9} CFU/ml at the 12th hour before decreasing to 1.62×10^{9} CFU/ml at the 24th hour. A temperature of 35°C showed the optimum condition for *C. kochii* (GSD) bacteria with a density of 3.93×10^{9}CFU/ml at 24 hours. These results suggest *C. kochii* (GSD) is categorized as mesophilic bacteria. Research on the growth of *E. coli* O157:H7 has shown that populations grown at 37°C are more resistant to heat treatment than those grown at 23°C and 30°C (Kim *et al*., 2019). At 40°C, there was an increase in the initial density of 3.32×10^{9} CFU/ml, which decreased to 3.25×10^{9} CFU/ml at the end of the experiment. The reduction in amount was primarily due to thermal stress on *C. kochii* (GSD) bacteria. Elshazly *et al*. (2023) stated that higher temperatures could destroy metabolic activity, thus affecting the growth process because of enzyme damage. At the

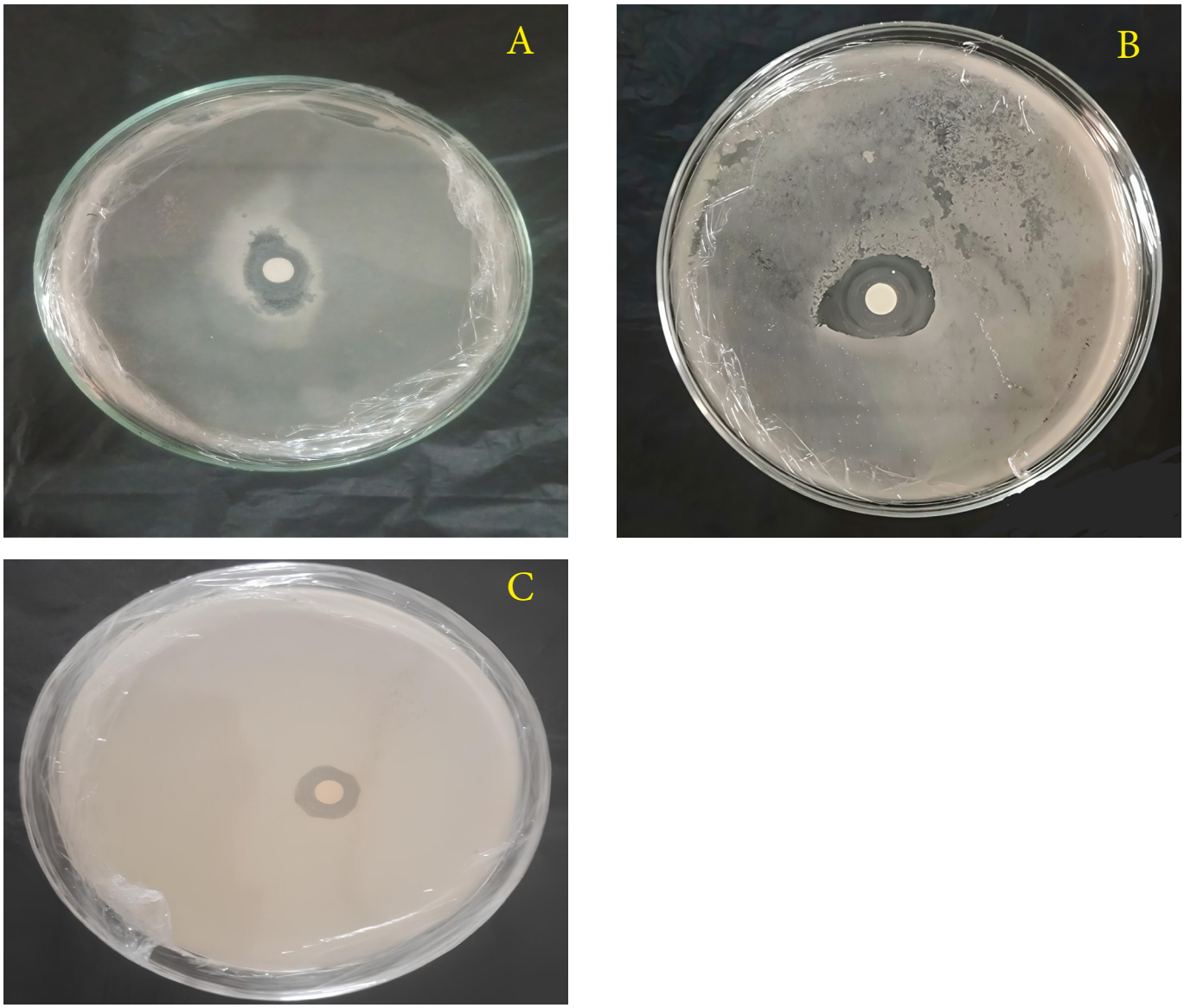

Figure 2. The results of fucoidanase enzyme activity assay in (a) temperature, (b) pH, (c), and salinity on growth *C. kochii* GSD.

highest temperature of 43.4°C, bacterial growth increased to 4.07×10^9 CFU/ml by the 15th hour. However, it declined to 3.60×10^9 CFU/ml by the 24th hour, indicating the limit of bacterial tolerance to high temperatures.

Five different pH conditions, pH 3.6, 5, 7, 9, and 10.3, were used in the study. The results showed that pH optimum affected the growth of *C. kochii* (GSD) and showed different growth patterns. Table 4 shows that the peak cell density, from lowest to highest, was found at pH 3.6, pH 5, pH 10.3, pH 9, and pH 7. At pH 3.6, the bacterial growth was relatively stable and reached the highest growth at the 9th hour of 0.527×10^8 CFU/ml, while at pH 5, the highest cell density at the 9th hour was 3.89×10^9 CFU/ml. At pH 10.3, the peak cell density was measured at 2.78×10^8, with growth in the next hour quite stable. The growth at pH 10.3 was slightly lower than pH 9, which reached 3.80×10^9 CFU/ml. pH 7 showed the highest cell density of about 3.64×10^9 CFU/ml. The growth of bacteria at pH 7 was quite high and stable, which shows that this condition is the most optimal for bacterial growth. This result is under the confirmation of Barzkar *et al.* (2023) that fucoidanase enzyme-producing bacteria can grow optimally at pH 6.5-9.1. The role of pH is significant for bacteria because it affects the acceleration of enzymes in catalyzing important reactions for bacteria. The concentration of hydrogen ions directly influences the three-dimensional structure of enzymes, thereby affecting their activity and growth. Each en zyme has an optimal pH. *C. kochii* (GSD) bacteria can grow optimally at pH 7 because the three-dimensional structure is most suitable for binding substrates. If the hydrogen ion concentration deviates from its optimal

concentration, the enzyme activity gradually decreases until the enzyme becomes non-functional (Sun *et al*., 2020). These results suggest that a neutral pH supports rapid growth and preserves cell stability over an extended period, enabling optimal growth under neutral conditions. The effect of incubation time shows the adaptation of bacteria to the environment with changes in metabolic activity during longer incubation periods. Different responses were observed at each pH level as time increased, resulting in different growth patterns over the 48 hours of observation. This adaptation is consistent with bacterial growth patterns that depend on varying pH (Zárate-Chaves *et al*., 2013).

Besides temperature and pH, salinity also affects the growth of *C. kochii* GSD. Table 2 shows the total density of *C. kochii* GSD at various salinities (3.5 ppt, five ppt, 17.5 ppt, 30 ppt, and 38.5 ppt) during 24 hours of observation. In 3.5 ppt salinity media, the number of bacterial cells hit its highest point at the 12th hour (3.62×10^9 CFU/ml) and declined significantly until the 21st hour. A salinity of 5 ppt showed relatively stable growth, with a peak at hour 21 (2.73×10^9 CFU/ml) and a slight decline at hour 24. In 17.5 ppt salinity media, bacterial growth peaked at hour 21, reaching 3.68×10^9 CFU/ml before slightly declining by hour 24. The salinity level of 30 ppt resulted in the highest growth, reaching 3.92×10^9/ CFU/ml at the 21st hour, and remained relatively high throughout the experiment. The 38.5 ppt salinity medium showed a similar pattern, with a peak at the 18th hour (3.67×10^9 CFU/ml) and a decrease at the 24th hour. Overall, 30 ppt salinity supported more stable and optimum bacterial growth than other salinities. Exposure to varying salinity levels can significantly impact bacterial growth rate, the number of generations, and generation time (Zainuddin *et al*., 2022). *C. kochii* GSD is a bacterium from the family of Bacillaceae and is classified into the mild halophilic category. Based on research conducted by Seiler *et al*. (2012), they found that bacteria from the Bacillaceae family, namely *Bacillus kochii*, are halophilic bacteria that require certain minimum salinity concentration levels. The optimum salinity that supports bacterial growth is varied: 20-50 ppt for mild halophilic bacteria, 50-200 ppt for moderate halophilic bacteria, and 200-300 ppt for extreme halophilic bacteria (Arisandi *et al*., 2017). Bacteria that can live in high salinity will adapt to the existing osmotic pressure (Zainuddin *et al*., 2022).

3.2.2 Fucoidanase enzyme activity assay

The qualitative enzymatic activity assay of fucoidanase aims to evaluate the activity of fucoidanase by observing the formation of clear zones. These clear zones indicate that the bacterium *C. kochii* GSD can degrade fucoidan in its growth medium (Setyawan *et al*., 2023). The hydrolysis process was monitored under optimal environmental conditions to maximize the production of fucoidanase. During a 96-hour observation period, the highest activity was observed at 72 hours, as evidenced by clear zones measuring 31.17 mm (temperature), 25.9 mm (pH), and 20.97 mm (salinity). These results are consistent with those of Setyawan *et al*. (2023), who also reported peak enzymatic activity at 72 hours. The measurements of fucoidanase activity are presented in Table 5. The optimal growth of fucoidanase-producing bacteria at specific time points, such as 72 hours, is influenced by several factors, including enzyme induction, nutrient availability, and metabolic adaptations. Studies have shown that bacteria, such as those from the genus *Vibrio* or *Formosa* algae, produce fucoidanase and related enzymes in response to the presence of fucoidan in the medium.

These enzymes are typically induced as bacteria metabolize fucoidan as a carbon source, with maximal activity observed during specific growth phases. For instance, one study highlighted that fucoidanase production peaked after an initial lag phase, reflecting the time required for bacterial adaptation and enzyme synthesis. Additionally, environmental factors such as pH, temperature, and ions like Mg^{2+} and Ca^{2+} significantly enhance enzymatic activity, aligning with the observed 72-hour peak in this study (Silchenko *et al*., 2018; Liu *et al*., 2023). CPC, a cationic quaternary ammonium compound with the empirical formula ($C_{21}H_{38}NCl$), interacts with the negatively charged phospholipid bilayer of bacterial cell membranes, disrupting cell integrity. CPC also exhibits unique properties when reacting with alginate or fucoidan, producing a white coloration. However, CPC does not respond to hydrolyzed fucoidan, resulting in a colorless zone in areas where enzymatic digestion has occurred (Sawant *et al*., 2015).

These clear zones indicate fucoidan degradation by the fucoidanase enzyme produced by *C. kochii* GSD. The use of CPC facilitated the degradation process (Naoe *et al*., 2020) a peak clear zone size of 31.17 mm at 72 hours. A slight decrease to 30.85 mm at 96 hours likely resulted from enzyme instability or substrate depletion affecting bacterial viability. Anisha *et al*. (2022) explained that fucoidan's anionic properties, due to its negatively charged sulfate ester groups ($-OSO_3^-$), interact electrostatically with positively charged molecules like proteins, enhancing its biological activity. Proteins in the medium are crucial for bacterial growth. Silchenko *et al*. (2018) state that enzymatic activity predominantly correlates with bacterial biomass. Bacterial growth diminishes over time due to temperature interactions, carbon-to-nitrogen

ratios, and oxygen availability (Sahu *et al*., 2019). *Flavobacterium algicola* produces fucoidanase most effectively at 40°C, maintaining stable activity within a temperature range of 25–55°C and at a pH of 9 (Qiu *et al*., 2022). Similarly, Liu *et al*. (2023) observed *Cobetia amphilecti* generates fucoidanase at an optimal temperature of 30 °C and pH 8. Deviations from optimal pH can lead to enzyme denaturation, while optimal conditions promote enzyme stability and higher catalytic activity (Dumorné *et al*., 2017).

At a salinity of 30 ppt, the largest clear zone observed was 20.97 mm at 72 hours, suggesting this condition supports the optimal activity of fucoidanase produced by *C. kochii* GSD. The salinity level reflects the natural marine habitat of the bacterium, where osmotic pressure maintains cell membrane stability and enzymatic activity. However, the clear zone size decreased slightly to 20.23 mm at 96 hours, potentially due to the accumulation of fucoidan degradation products, such as fucoidan oligosaccharides, acting as enzyme inhibitors. Kuddus (2019) explained that inhibitors can interact with enzymes to reduce their catalytic activity, with competitive inhibition likely occurring in this case. This decrease could also be attributed to changes in the microenvironment, such as reduced nutrient availability or altered pH from bacterial metabolic activity during incubation. Despite the observed decline, the reduction in activity was relatively small, indicating that fucoidanase remains active even as its activity begins to wane. Under these conditions, a salinity of 30 ppt continues to provide optimal support for fucoidanase activity, as osmotic pressure stabilizes enzyme structure and catalytic function. Salinity regulation is critical for maintaining bacterial cell turgor pressure and metabolic function. Yuliani *et al*. (2018) noted that marine bacteria require specific osmotic conditions to avoid lysis or cellular dehydration. Thus, a salinity of 30 ppt can be considered optimal for fucoidanase activity and bacterial growth.

4. Conclusion

Sargassum polycystum powder was extracted and used to synthesize fucoidan material and assay the activity of fucoidanase enzyme-producing bacteria. In the environmental test experiment, the optimum conditions for the growth of *C. kochii* GSD were temperature 35, pH 7, and salinity 30 ppt, respectively. Proper temperature, pH, and salinity regulation are crucial for large-scale fucoidanase production, such as in industrial fermentation processes utilizing marine bacteria to produce enzymes efficiently. Enhancing fucoidan degradation efficiency by optimizing temperature, pH, and salinity levels can lead to the production of high-value, low-molecular-weight fucoidan. This research provides the first comprehensive optimization study of a tropical fucoidanase-producing bacterium, filling a crucial research gap in microbial bioprocessing. Future studies should focus on scaling up enzyme production for industrial applications and evaluating the bioavailability of LMWF in aquatic organisms.

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Authors' Contributions

All authors have contributed to the final manuscript. Each author's contribution is as follows: AS, MKA, HPF, AFL, MWY: conception and design of the study and wrote the manuscript. AAN, RHS, WAP: performed the experiment, collected the data, and interpreted the data. NLGRJ: supervised the manuscript. ARR and SS: critically revised the article. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

Declaration of Artificial Intelligence (AI)

The authors affirm that no artificial intelligence (AI) tools, services, or technologies were employed in the creation, editing, or refinement. All content presented is the result of the independent intellectual efforts of the author(s), ensuring originality and integrity.

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