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# Cellulase Activity of Rizosfer Bacteria of Ketapang Plants (*Terminalia catappa* L.)

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## Abstract

*Cellulose, a natural biopolymer produced by plants, undergoes enzymatic breakdown by cellulase, cleaving the monomer's β-1,4 glycosidic bonds. Cellulolytic bacteria, producers of cellulase, can be isolated from plant rhizospheres, like Ketapang (Terminalia catappa L.), abundantly found in the Biology Department at Universitas Sriwijaya, South Sumatra, Indonesia. This study aimed to isolate cellulolytic bacteria from Ketapang rhizosphere soil and assess their cellulase activity. The spread plate method on selective Carboxy Methyl Cellulose Agar (CMC agar) was used for bacterial isolation, and cellulase activity was measured using the DNS method (3.5-dinitro salicylic acid). Results yielded 22 isolates of cellulolytic bacteria capable of growth on selective CMC agar. Thirteen isolates, identified through a screening test, produced cellulase, forming clear zones post-addition of 0.1% Congo Red and 1 M NaCl. Cellulolytic activity indices for these isolates ranged from 0.02 to 2.6 mm. The highest indices (IAS) and cellulase activities were observed in BS10, BS4, and BS22, with IAS values of 2.64, 2.12, and 1.71, and cellulase activities of 354.99 U/ml, 9.42 U/ml, and 9 U/ml, respectively. Identification results suggested that isolate BS10 bore similarities to the genus Zoogloea, while isolates BS4 and BS22 showed similarities to the genus Bacillus.*

*Keywords : Cellulase, Cellulolytic Bacteria, Rhizosphere, Carboxy Methyl Cellulose, DNS method.*

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## 1. Introduction

Cellulose, the primary component of plant cell walls, is a lengthy glucose polymer linked by β-1,4 glucoside bonds. Enzymatic hydrolysis, facilitated by the cellulase enzyme, breaks these bonds into glucose monomers [1]. Cellulolytic bacteria, a type of bacteria, produce cellulase enzymes crucial for hydrolyzing cellulose into the simpler product, glucose [2]. Cellulolytic bacteria synthesize a set of enzymes, including 1,4-β-endoglucanase, 1,4-β-exoglucanase, and β-glucosidase, which collaborate in the hydrolysis of cellulose [3] [4]. These bacteria are recognized for their robust bacterial growth, resilience in the challenging conditions of the bioconversion process, stability, and the presence of multi-enzyme complexes. This enhances their functionality and synergy, making them valuable producers of potent cellulase enzymes [5].

The cellulase enzyme, produced intracellularly and subsequently released into the growth medium, can utilize cellulose as a carbon source in metabolic processes [6] [7]. Cellulase enzymes from cellulolytic bacteria find extensive applications in various industries, including the textile industry for softening cotton and finishing denim, as well as in the pulp and paper industry for fiber modification [8]. Additionally, they are used in the detergent industry for dye treatment and cleaning processes [9].

Cellulolytic bacteria primarily inhabit the rhizosphere in soil, known for intense microbial activity and considered the most complex soil environment [10]. The rhizosphere, influenced by plant roots, is where roots release organic compounds, including sugar, amino acids, vitamins, cellulase enzymes, and more, into the soil [11]. Root exudates, serving as the main food and energy source for cellulolytic

bacteria in the rhizosphere, contribute to increased population and activity, facilitating the swift breakdown of organic material.

Ketapang plants, also known as tropical almonds (Terminalia catappa L.), are frequently found on the Srwijaya University campus, particularly around the Biology department in the Faculty of Mathematics and Natural Sciences, Indralaya, South Sumatra. Belonging to the Combretaceae family, Ketapang is a large wild tree [12]. As a natural resource, cellulose, the most abundant natural polymer, is present in nature [13].

Research on cellulolytic bacteria producing cellulase enzymes in plant rhizospheres has been conducted. Notably, a study [14] successfully identified Bacillus brevis as a cellulolytic bacteria in the rhizosphere of Aquilaria malaccensis, displaying effective cellulose hydrolysis activity. However, no research on cellulolytic bacteria in the rhizosphere of Ketapang plants has been found. Therefore, there is a need to investigate cellulolytic bacteria producing cellulase enzymes in the rhizosphere of Ketapang plants, considering their abundance as natural resources around the campus of the Department of Biology, Faculty of Mathematics and Natural Sciences, Sriwijaya University, Indralaya, South Sumatra.

## 2. Materials and Methods

### 2.1 Sampling

Soil samples were collected in the rhizosphere of Ketapang plants around the campus of the Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Sriwijaya, Indralaya, South Sumatra (Figure 1). Utilizing purposive sampling, composite sampling was conducted in the rhizosphere of six Ketapang plants, extracting soil around the roots to a depth of 0-20 cm. The collected soil was aseptically placed in plastic bags, stored in a cool box, and transported to the laboratory, where it was refrigerated [15].

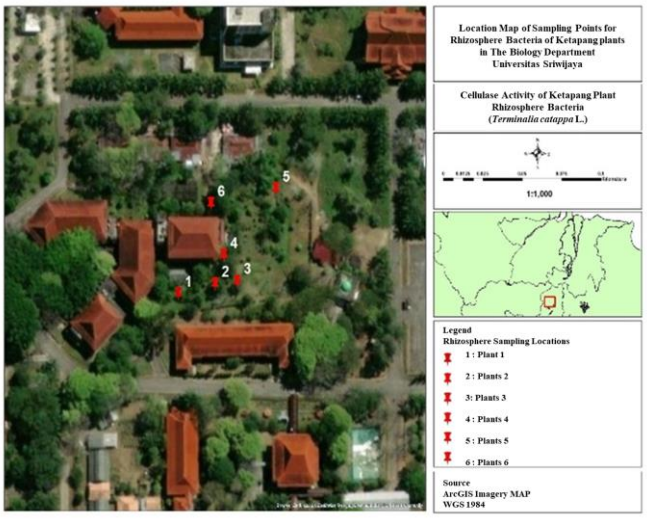


Figure 1. Sampling location

### 2.2 Media Creation Procedures and Sterilization

The cellulolytic bacteria isolation utilized CMC agar media, comprising carboxymethylcellulose (CMC) 0.5%, NaNO3 0.1%, K2HPO4 0.1%, KCl 0.1%, MgSO4.7H2O 0.05%, yeast extract 0.05%, and agar 2% [16]. These ingredients were weighed, placed in an Erlenmeyer flask, dissolved in distilled water, and the pH was adjusted to 7 using acid or base solutions. The mixture was then heated until dissolved on a magnetic stirrer hot plate and sterilized at 121°C under a pressure of 15 lbs for 15 minutes [9].

### 2.3 Isolation and Purification of Cellulolytic Bacteria

Isolation involved serial dilution of a 10-gram sample in a 90 mL physiological salt solution in an Erlenmeyer flask to create a bacterial suspension ($10^{-1}$). After homogenization, dilution series from $10^{-1}$ to $10^{-5}$ were performed. The last three dilutions ($10^{-3}$, $10^{-4}$, and $10^{-5}$) were plated by spreading 100 µl of each dilution onto solid CMC media using the spread plate method. Incubation at room temperature for 48 hours followed. Purification of growing isolates involved selecting different colonies based on shape, color, and size. These selected colonies were streaked onto NA media plates using the quadrant scratch plate method and incubated for 2 days at room temperature. Colonies displaying single growth were considered pure isolates. These single colonies were picked, inoculated into slanted NA media, and then incubated at room temperature for 48 hours before storage in the refrigerator.

### 2.4 Cellulolytic Bacterial Screening

The cellulolytic bacterial isolate was inoculated onto solid CMC agar media by dripping it onto the agar, followed by incubation at room temperature for 48 hours. A screening test ensued, flooding the petri dish with a 0.1% Congo Red solution for 15 minutes. After discarding the Congo Red solution, the petri dish was washed with 1M NaCl solution and left to stand for 15-20 minutes. The formed clear zone indicated the bacterial isolate's cellulose degradation capability. This clear zone's diameter was measured with a ruler to determine the cellulase activity index, a qualitative test of cellulase activity. The cellulase enzyme production ratio is expressed as the clear zone diameter to the colony diameter ratio, calculated using the following formula [17]:

$$\text{Cellulase activity index} = \frac{\text{czd} - \text{bcd}}{\text{bcd}}$$

Note:
czd = clear zone diameter
bcd = bacterial colony diameter

The cellulase activity index values convey the reaction strength: ≥ 2 signifies a strong reaction, 1-2 indicates a moderate reaction, ≤ 1 denotes a weak reaction, and a value of 0 implies no reaction at all [18]; [19].

### 2.5 Preparation of Standard Inoculum for Cellulolytic Bacteria

Isolates demonstrating qualitative cellulase activity are transformed into standard inoculum for these bacteria. The standard inoculum is prepared by inoculating 1 dose of the isolate into 20 ml of sterile liquid CMC media, comprising 1% CMC, 0.1% NaNO3, 0.1% K2HPO4, 0.1% KCl, 0.05% MgSO4.7H2O, and 0.05% yeast extract, with the medium's pH adjusted to 7. After incubating at room temperature for 24 hours, the bacterial cell count in this inoculum is determined and adjusted to the standard inoculum number, specifically $10^6$ cells/ml [20].

### 2.6 Cellulase Crude Enzyme Extraction

Cellulase crude enzyme extraction was conducted in 250 ml Erlenmeyer flasks, each containing 95 ml of sterile liquid CMC medium with 1% CMC, 0.1% NaNO3, 0.1% K2HPO4, 0.1% KCl, 0.05% MgSO4.7H2O, and 0.05% yeast extract (pH 7), supplemented with 5% standard inoculum ($10^6$ cells/ml). The mixture was then incubated at room temperature with agitation at 130 rpm for 72 hours. Following incubation, the culture was centrifuged at 10,000 rpm for 10 minutes at 4 $^oC$ to obtain the supernatant, which served as the crude enzyme extract for cellulase activity testing [16].

### 2.7 Preparation of a Glucose Standard Curve

To quantitatively determine cellulase activity, a glucose standard curve was generated using the DNS method. The concentration of reducing sugar in the glucose standard curve was determined using the DNS method, with a prepared standard glucose solution featuring concentrations of 0, 20, 40, 60, 80, and 100 mg/L. In each test tube, 1 ml of the standard glucose solution was combined with 1 ml of DNS solution, homogenized, boiled for 15 minutes, and then cooled after turning brick red. To measure the concentration, 1 ml of 40% KNa-Tartrate solution was added, and the absorbance of each concentration series was measured at a wavelength of 540 nm using a UV-Vis spectrophotometer. The obtained absorption values were used to construct a glucose standard curve with the line equation y = ax + b, which is then employed to determine the glucose concentration (x) of the sample [21].

### 2.8 Cellulase Activity

Cellulase activity was evaluated through the DNS method, employing prepared reaction tubes for samples, blanks, and controls. In the sample tube, 0.5 ml of crude enzyme extract was mixed with 1 ml of 1% CMC (prepared in 50 mM citrate buffer, pH 5) and 0.5 ml of 50 mM citrate buffer, pH 5. The mixture underwent a 30-minute incubation at 50°C in a water bath, followed by the addition of 1 ml of DNS solution to halt the reaction and boiling for 15 minutes. Simultaneously, the same procedures were applied to blanks and controls. The blank used a pH 5 citrate buffer solution without enzymes, reacting with the substrate. In the control treatment, the enzyme was first inactivated by heating for 15 minutes in boiling water before reacting with the substrate. The absorbance of samples, controls, and blanks at a wavelength of 540 nm was measured using a UV-Vis spectrophotometer. Cellulase activity, measured in units of µmol glucose units released in 1 minute, was calculated using a glucose standard curve, with levels determined by the formula [22]:

$$\text{Cellulase activity (U/ml)} = \frac{\text{sgc} \times 1000}{\text{V. t. BM}}$$

A = ((As - Ab) - (Ak - Ab))

Note:
As= Sample absorbance
Ab= Blank absorbance
Ak= control absorbance
V= Enzyme volume
Sgc=sample glucose concentration
t= Incubation time
BM= molecular weight of glucose
1000= conversion from mmol to µmol

### 2.9 Identification of Cellulolytic Bacteria

The three selected cellulolytic bacterial isolates, exhibiting high cellulolytic index values, underwent characterization and identification. Macroscopic characterization involved assessing colony morphology, encompassing shape, color, edges, and elevation. Microscopic characterization included cell morphology, involving gram staining (cell shape and gram characteristics) and endospore staining. Biochemical characterization involved oxygen demand, catalase, MIU (motility/indole/urease), citrate, sugar fermentation, methyl red, Voges-Proskauer (VP), triple sugar iron, agar, gelatin, and starch hydrolysis tests. Identification was conducted using Bergey's Manual of Determinative Bacteriology 9th.

## 3. Results and Discussion

From the conducted research, 22 cellulolytic bacterial isolates were successfully obtained from the rhizosphere soil of Ketapang plants surrounding the Biology Department campus, Faculty of Mathematics and Natural Sciences, Sriwijaya University, Indralaya, South Sumatra. Samples from six Ketapang plants were composited. CMC agar media was employed during the isolation stage, while Nutrient Agar media was used for purification. The outcomes of cellulolytic bacteria isolation and purification are presented in table 1.

Table 1. Cellulolytic Bacteria Isolation and Purification Results.

| Origin of isolate | Isolate Code |
|---|---|
| Rhizosphere of Ketapang plants | BS1, BS2, BS3, BS4, BS5, BS6, BS7, BS8, BS9, BS10, BS11, BS12, BS13, BS14, BS15, Bs16, BS17, BS18, BS19, BS20, BS21, BS22 |
| Total | 22 isolates |

Table 1 results indicate the presence of cellulolytic bacterial isolates in the rhizosphere soil of Ketapang plants, demonstrating their capability to degrade cellulose-containing substrates. This aligns with the assertion by [23] that cellulolytic bacteria possess the ability to break down cellulose-containing materials. As per [20], cellulolytic bacteria are characterized by their production of cellulase enzymes, utilized for the hydrolysis of cellulose into a source of energy and glucose.

Cellulolytic bacteria are typically located in the rhizosphere soil, the environment supporting plant root growth and showcasing heightened microbial activity [24]. The quantity of bacteria in the rhizosphere significantly differs from surrounding areas, as highlighted by [11], owing to the presence of root exudates. These exudates serve as the primary source of food and energy for rhizosphere bacteria. Plant roots release diverse exudates, including organic compounds, sterols, enzymes, proteins, hormones, and other substances. The organic compounds in root exudates encompass sugars, amino acids, amides, aliphatic acids, phenols, and fatty acids. This aligns with [25], stating that the presence of root exudates contributes to an increased population of rhizosphere bacteria.

Twenty-two cellulolytic bacterial isolates underwent screening to assess their cellulose degradation ability, evident through the formation of clear zones around bacterial colonies. The screening results revealed 13 isolates proficient in cellulose degradation, as illustrated in Figure 2.

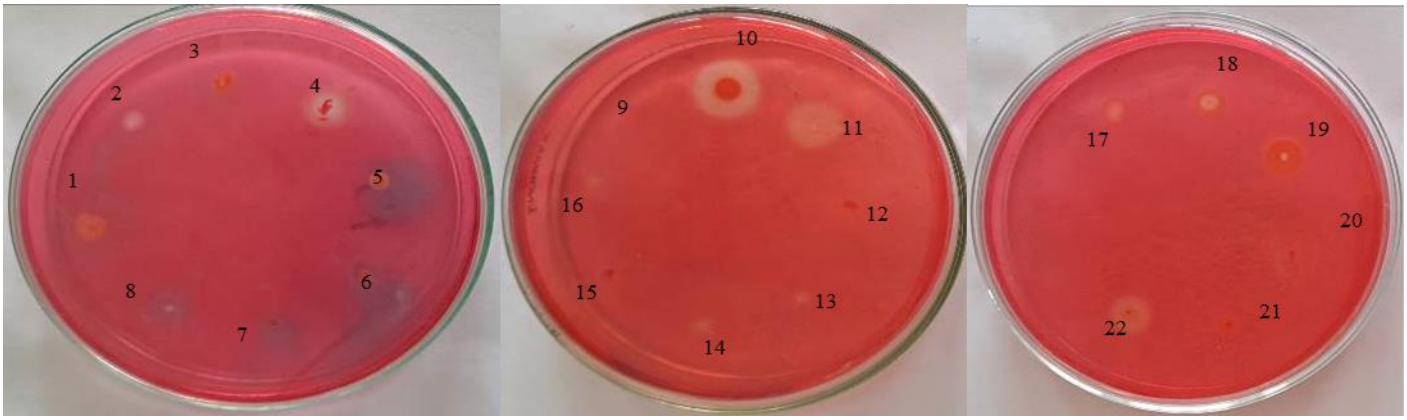


Figure 2. Screening test results for cellulolytic bacterial isolates

Description: 1=BS1; 2=BS2; 3=BS3; 4=BS5; 5=BS6; 6=BS6; 7=BS7; 8=BS8; 9=BS9; 10=BS10; 11=BS11; 12=BS12; 13=BS13; 14=BS14; 15=BS15; 16=BS16; 17=BS17; 18 =BS18; 19=BS19; 20=BS20; 21=BS21; 22=BS22

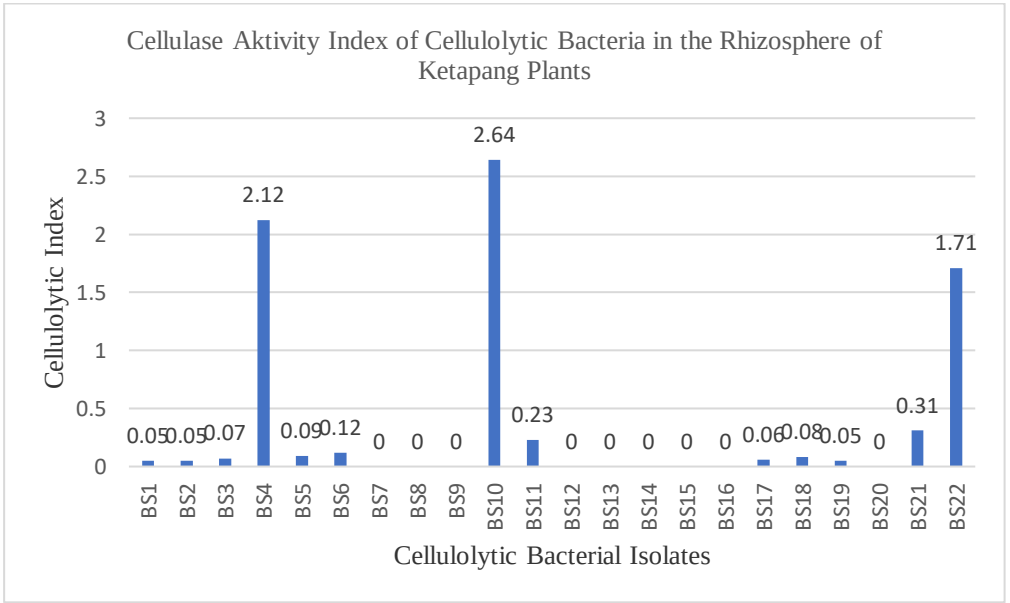


Figure 3. Cellulase activity index of cellulolytic bacteria in the rhizosphere of Ketapang plants

Figure 2 illustrates the screening results for cellulolytic bacteria using 0.1% Congo Red and NaCl solution. Among the isolates, 13 demonstrated the capability to degrade cellulose, while 9 were unable to do so. The degradation of cellulose in the CMC substrate is evidenced by the formation of a clear zone. This occurs because Congo Red colors the cellulose in the CMC medium, while degraded cellulose by cellulolytic bacteria remains uncolored [19]. Additionally, the reaction with benzidindiazo sodium salt-bis-1-naphthylamine-4-sulfonate (Congo Red) strongly interacts with β-1,4-glycosidic bonds in CMC [26]. Media with broken β-1,4-glycosidic bonds do not stain, resulting in the formation of a clear zone [27].

The research identified 13 isolates capable of producing clear zones with cellulase activity index values (Figure 3). These isolates, namely BS1, BS2, BS3, BS4, BS5, BS6, BS10, BS11, BS17, BS18, BS19, BS21, and BS22, exhibited varying cellulase activity index values. Isolates BS1, BS2, BS3, BS5, BS6, BS11, BS17, BS18, BS19, and BS21 had values ranging from 0.02 to 0.3, while isolate BS22 showed a value of 1.71, and isolates BS4 and BS10 demonstrated values of 2.12 and 2.64, respectively. The diverse cellulase activity index values among isolates are attributed to both internal factors (genes) and external factors (nutrition, temperature, and incubation time), as noted by [28].

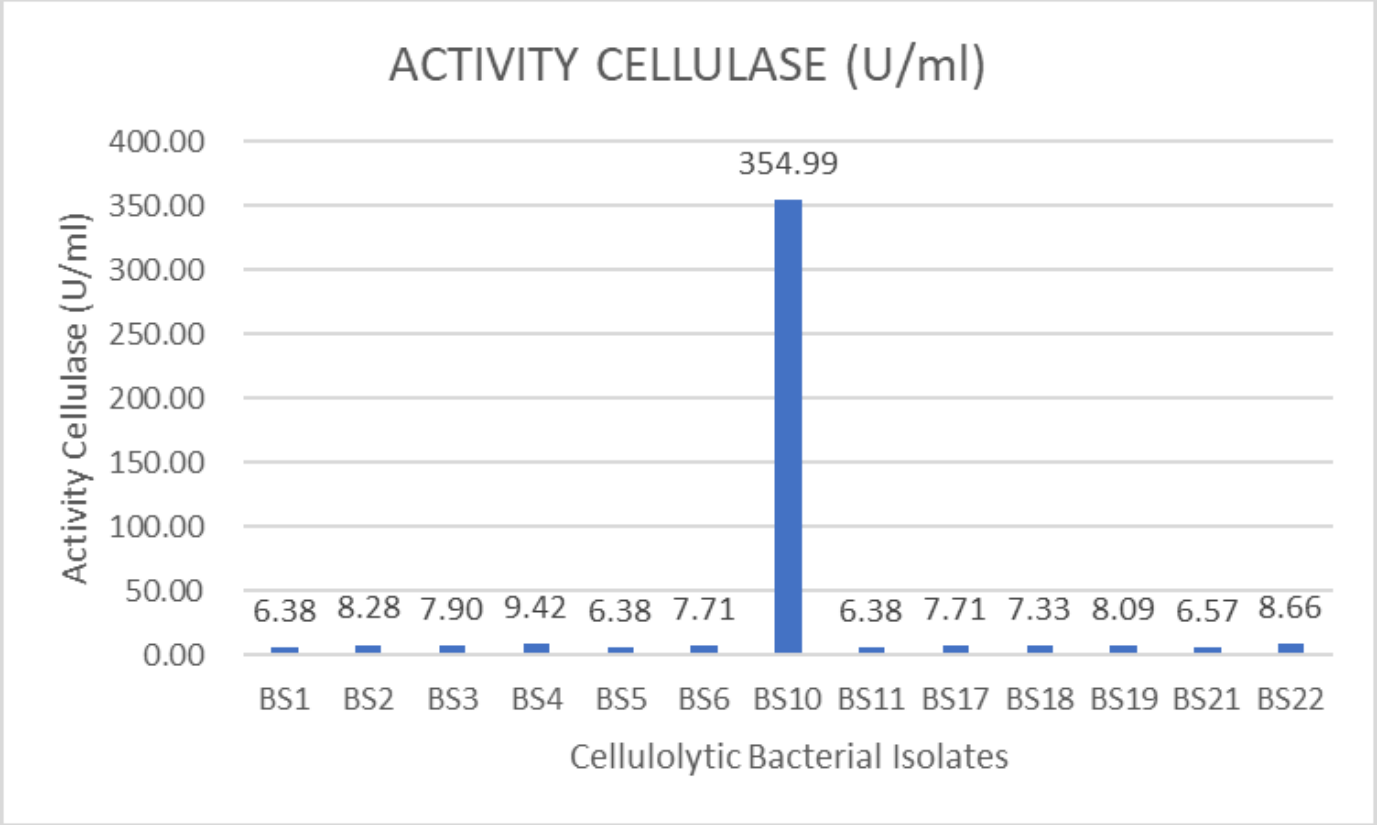


Figure 4. Cellulase activity of cellulolytic bacteria in the rhizosphere of Ketapang plants

Based on the Cellulolytic Activity Index (IAS) category as defined by [18], isolates BS10 and BS4 exhibit a strong reaction (IAS ≥ 2), while isolate BS22 falls into the moderate reaction category (IAS 1-2). Ten isolates display a weak reaction (IAS ≤ 1), and nine isolates show no cellulolytic reaction at all (IAS=0). The cellulolytic activity index is a crucial metric for cellulolytic bacteria, reflecting their cellulose-degrading ability, as emphasized by [21]. Bacterial species with a high cellulolytic activity index can produce substantial quantities of cellulase enzymes.

Three isolates, namely BS4, BS10, and BS22, exhibiting high cellulase activity indices, were chosen for subsequent cellulase activity testing. The results showed that the cellulase activity for the BS4 isolate was 9.42 U/ml, BS10 isolate was 354.99 U/ml, and BS22 isolate was 8.66 U/ml (Figure 4). Among these, BS10, BS4, and BS22 had the highest initial cellulase activity values. The IAS value for the BS4 isolate did not correlate with the cellulase activity value, in contrast to the BS10 isolate, where a strong reaction corresponded to high cellulase enzyme production. This discrepancy in enzyme production between the IAS value

and the activity value for BS4 is attributed to differing bacterial environmental conditions between solid and liquid medium cultures during testing, influencing enzyme production [29] [22].

Table 2. Morphological and biochemical test results of BS4, BS10 and BS22 cellulolytic bacterial isolates

| Characteristics | | Isolate | | |
|---|---|---|---|---|
| | | BS4 | BS10 | BS22 |
| Morphology Test | Colonies on NA medium Upright | Enchilate | Filiform | Enchilate |
| | Colonies on NA Slant medium | Echinulate | Beaded | Echinulate |
| | Form | Irregular | Irregular | Irregular |
| | Elevation | Effuse | Raised | Effuse |
| | Edge | Ciliates | Undulate | Ciliates |
| | Color | Beige | White | Beige |
| | Cell shape | Bacilli | Bacilli | Bacilli |
| | Gram staining | Positive | Negative | Positive |
| | Endospores | Endo-spore forming | Endo-spore not forming | Endo-spore forming |
| Biochemical Test | Oxygen requirement | Facultatively anaerobic | Facultatively anaerobic | Facultatively anaerobic |
| | $H_2S$ (TSIA) | - | - | - |
| | Motility | + | + | + |
| | Methyl red | + | - | + |
| | Voges proskauer | - | + | - |
| | Simmons citrate | - | - | - |
| | Starch | - | - | - |
| | Gelatin | + | + | + |
| | Catalase | + | - | + |
| | Indol | - | - | - |
| | Urea hydrolysis | - | - | - |
| | Casein hydrolysis | + | + | + |
| | Glucose hydrolysis | - | + | - |
| | Sucrose hydrolysis | - | + | - |
| | Lactose hydrolysis | - | + | - |
| | Genus | *Bacillus* | *Zoogloea* | *Bacillus* |

Description: (+): means positive; (-): means negative

Cellulase activity was assessed by quantifying reducing sugar levels through the DNS method, utilizing a glucose standard curve. The DNS method is commonly used to measure reducing sugar products resulting from enzymatic reactions involving polysaccharide-degrading enzymes. In this method, reducing sugars react with the 3,5-dinitrosalicylic acid (DNS) reagent, producing a brownish-yellow compound identified as 3-amino-5-nitrosalicylic acid [22, 30].

The three cellulolytic bacterial isolates underwent identification for determining their genus, focusing on their ability to degrade cellulose. Characterization, including morphological and biochemical observations, is presented in Table 2. Based on these results, isolates BS4 and BS22 exhibited similarities to the genus *Bacillus*, while BS10 showed similarities to the genus *Zoogloea*. Comparable studies by [14] identified *Bacillus brevis* in the rhizosphere of *Aquilaria malaccensis*, and [31] discovered the genus *Flavobacterium* in the rhizosphere of oil palm.

## 4. Conclusion

Three cellulase-producing bacterial isolates were identified: *Bacillus* sp1 (BS4), *Bacillus* sp2 (BS22), and *Zoogloea* (BS10). Their respective cellulase activities were measured at 9.42 U/ml, 8.66 U/ml, and 354.99 U/ml.

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