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Article

Cellulolytic Bacteria Isolation from Sugarcane Garden Soil

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Arief Muammar[1]*, Mesha Arjuna[1], Maria Manullang[1], Endah Retnaningrum[1]

[1]Department of Tropical Biology, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia

Abstract. The soil surrounding the sugar cane garden is a promising habitat for cellulolytic bacteria because it contains a source of cellulose, hemicellulose, and other carbon sources derived from the sugar canes remaining organic matter. This study attempts to determine the presence of cellulolytic bacteria isolated from sugar cane garden soil, as well as the ability of cellulolytic bacteria to degrade cellulose based on optical density and sugar reduction values isolated from sugarcane garden soil. We collected soil samples from five points (T1, T2, T3, T4, T5), isolating the bacteria in CMC (Carboxyl Methyl Cellulose) medium, qualitatively testing the cellulase enzyme activity with Congo Red and Iodine Assays, and quantitatively using the DNS Assay. The results of the qualitative test with the Congo Red assay revealed that there were three positive isolates with a clearly visible clear zone, namely TII C4, TII C1, and TIV C4, and after quantitative supernatant tests, the OD values of all isolates were higher than the negative control in supernatant samples using 540nm wave length using spectrophotometer, by using synthetic cellulose called Carboxy Methyl cellulase (CMC) as a substrate in the cellulase enzyme test.

Corresponding Author :
Arief Muammar
Department of Tropical Biology, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia
Email: arief.muammar@ugm.ac.id

1. Introduction

Cellulose is a linear polymer structure containing D-glucose linked in 1,4 glycosidic linkage. Besides cellulose, the major composition of lignocellulose, which is important to make physical strength in cell walls and other parts of plants, also consists of hemicellulose and lignin [1]. With that complex structure, lignocellulose hydrolysis to creates its monomer, glucose, will need a lot energy.

One of the application using cellulose nowadays is creating bioethanol using cellulose as a carbon source cellulo-ethanol. There is a common problem faced by companies thar create this kind of ethanol. That was in the pretreatment processes, the company needed to add cellulase enzyme at very expensive price. But now researchers have found the strategy to solve this problem. The production price to buy cellulase enzyme could be cut off if we could make those cellulase enzymes by ourselves using genetic modification of microorganisms. To be able to produce cellulase by ourselves, we need to find several microorganisms that have those cellulase genes and efficiently convert cellulose to become glucose. We need to isolate those genes and express them in yeast to produce ethanol. One of examples of those miccroorganisms that have those genes was cellulolytic bacteria [2-3].

Cellulolytic bacteria are one of the microorganisms that can degrade cellulose. Those microorganisms could use cellulose as a carbon source in their metabolism. Conventionally, cellulolytic bacteria can be used as agents to make products such as bioethanol, organic chemicals, and good-quality nutrition sources for animal feed and food sources [4]. Comparative studies indicate clear trade-offs: commercial enzyme blends provide predictable performance but remain expensive and sensitive to inhibitors; heterologous expression in yeasts can lower cost but often struggles with secretion, folding, and glycosylation limitations under industrial conditions; and cellulolytic bacteria enable consolidated hydrolysis but vary widely in activity, stability, and compatibility with process pH, temperature, and inhibitor profiles. These differences underscore the practical need for new, cost-effective enzyme sources and for screening frameworks that identify robust cellulolytic candidates suitable for gene mining and downstream expression.

Cellulolytic bacteria in nature are found in various ecosystems. Croplands, peatlands, the rumen of ruminant animals, the intetsine of insects, the gastrointestinal of humans, litter of leaves, slurry, and others bacterial habitats [5-10]. Litter leaf that accumulated in the sugarcane garden soils is one environment that consists of a lot of cellulose and its decomposer [11], but it has not been studied much until now. However, findings across studies are difficult to compare critically because screening practices are heterogeneous: some rely on plate-based halos on carboxymethyl cellulose with Congo red staining, others report enzyme activities (CMCase, FPase, β-glucosidase), and still others employ growth-centric metrics such as optical density (OD) in defined cellulose media [12-13]. As a result, performance claims from well-studied niches (e.g., rumen, compost) do not translate directly to other habitats where isolates may face distinct physicochemical constraints.

Sugarcane garden soils represent a distinct, cellulose-rich niche. Leaf-litter accumulation (“trash”) provides sustained inputs of recalcitrant polysaccharides alongside fluctuating moisture and temperature, potentially selecting for decomposer communities with traits valuable for bioprocessing, such as thermotolerance and inhibitor resilience [14-15,]. Yet compared with rumen, compost, or forest litter, sugarcane garden soils remain under-sampled for cellulolytic bacteria, and most recent work in agricultural soils emphasizes community profiling rather than isolating strains and benchmarking their cellulose-degrading performance using standardized, quantitative criteria [16]. This lack of systematic, growth-based evaluation constitutes a clear research gap: there is limited isolate-level evidence from sugarcane soils that directly links growth on cellulose to candidacy for enzyme characterization and gene isolation relevant to cost-effective bioethanol production [17-18].

Addressing this gap, the present study focuses on an underexplored agricultural habitat and applies a standardized, OD-based screening in defined cellulose media to generate directly comparable performance data across isolates. The novelty lies in combining targeted sampling of sugarcane garden soils with growth-centric benchmarking designed to prioritize strains whose cellulose utilization is robust under controlled conditions, thereby creating a practical shortlist for subsequent cellulase assays to reduce enzyme costs in lignocellulosic ethanol processes [19-21].

Immediately before the methods, we state our objective explicitly: to isolate cellulolytic bacteria from sugarcane garden soils and quantitatively evaluate their cellulose-degrading capacity using

optical-density measurements in defined cellulose media, identifying high-performing candidates for downstream cellulase characterization.

2. Experimental Section

2.1. Materials

A soil sampling was conducted at sugarcane garden in Berbah Subdistrict, Sleman, DI Yogyakarta, Indonesia. Then the sample was processed in the Microbiology Laboratory of the Faculty of Biology, Universitas Gadjah Mada for observing selection of cellulolytic bacteria in the sugarcane garden soil.

This research utilized various equipment, including analytical scales, test tubes, test tube rack, vortex, petri dish, bunsen, inoculation loop, eppendorf pipette, drygalski, incubator, mixer, laminar air flow, autoclave, refrigerator, stove, pan, erlenmeyer, micropipette, sticker label, zip lock plastic, centrifuge, and spectrophotometer.The materials which used in this research are soil samples from sugar cane garden, aquadest, ethanol, CMC medium, nutrient agar medium, NB medium, NaCl 1%, Congo Red 1%, CMC 1%, DNS Reagent, potassium phosphate buffer, Iodine solution and glass beads.

2.2. Methods

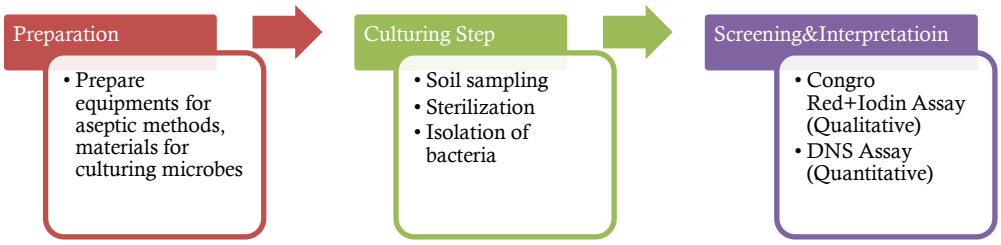

Figure 1. Research Flow

2.2.1. Soil Sampling

A soil sampling conducted at 5 sampling points (5 replications) located in the sugar cane garden, a ground floor mixed with sugar cane litter. The first point symbolized T1, the second point symbolized T2, the third point symbolized T3, and the fourth point symbolized T4 and the fifth point symbolized T5.

Soil samples which have been taken are stored in zip lock plastic and labeled according to sampling point symbol. Soil samples were brought to the Microbiology Laboratory of the Faculty of Biology, Universitas Gadjah Mada for further observation of the activity of cellulolytic bacteria. Samples are stored at room temperature.

2.2.2. Sterilization of Equipment and Material

Before equipments and materials are used, equipments and materials must be sterile. The equipments and materials washed with detergent. After that, all of them must be dried. Equipments which will be sterilized is wrapped with cotton and paper. After that, a sterilization method used autoclave to destroy microorganisms at 2 atm and 121°C for 1 hour.

2.2.3. Preparation of Soil Sample

Soil samples were taken one by one from zip lock plastic and each of them weighed 1 gram using an analytical scale. After that, it inserted into the test-tube with 9 ml of aquadest. Aquadest poured into the test tube using a 10 ml volume pipette that has been sterilized. The test tube contained soil samples and aquadest are homogenized with vortex until well blended. Then the soil sample is used 1ml with

an eppendorf pipette, put into another test tube which has filled with 9 ml aquadest and did dilution series. After that, homogenized with vortex again and carried out up to six-time dilutions.

2.2.4. Isolation of Bacteria

In test tube, each dilution is poured into a sterile petri dish of 1 ml of suspension using a dropper pipette. Nutrient CMC agar then poured 15 ml into a petri dish with contained microbial [22]. Petri dish is shaken horizontally in a clockwise direction, so bacteria can grow spreadly. Nutrient CMC Agar which has poured, waited until it was hard. Incubation of bacteria with an inverted position. Incubation of bacteria at 37ºC for 72 hours used an incubator. Bacteria's growth activities are carried out aseptically. After 72 hours of incubation, it was isolated from microbes which grew on the cellulose medium.

2.2.5. Qualitative Test of Cellulase Activity

The bacterial isolates were obtained from NB medium and inoculated onto CMC medium in petri dishes using the spread and dotting methods. After 48 hours of incubation at 37°C, the bacterial colonies were washed off with distilled water for observation. Cellulolytic activity was tested using the Congo Red method: 0.1% Congo Red solution was added, shaken, and removed, followed by 1% NaCl, which was spread and then removed. Clear zones indicated positive cellulolytic activity. Additionally, an iodine test was performed by inoculating bacteria on CMC medium, adding Gram's Iodine solution (0.133 g KI and 0.067 g I_2 in 20 ml distilled water), and rinsing after 3 minutes. Clear zones around colonies confirmed cellulase enzyme production.

2.2.6. Quantitative Test of Cellulase Activity

Bacterial isolates identified as cellulolytic from the Congo Red assay were further tested quantitatively using the DNS assay and spectrophotometry to measure OD values. Isolates were inoculated into 4 ml NB medium and incubated for one day. A 1% CMC solution was prepared in 10 mM potassium phosphate buffer (400 µl). The grown cultures were centrifuged to separate supernatants and pellets, with centrifugation repeated three times to collect all bacterial cells.

For the supernatant, 100 µl was mixed with the CMC-phosphate buffer solution and incubated at 45°C for 30 minutes. Then, 0.5 ml DNS reagent was added to 0.5 ml of the supernatant-CMC mixture, boiled for 5 minutes, and cooled. OD was measured at 540 nm using a spectrophotometer, with a blank solution (NB medium without sample) as a control. The sample's OD value was compared to the blank and a standard curve.

For the pellets, 0.1 ml glass beads and lysis buffer were added to lyse the cell wall, followed by vortexing and centrifugation at 13.000 rpm to obtain the cell lysate. From the supernatant, 100 µl was mixed with 400 µl CMC, and 0.5 ml DNS reagent was added to 0.5 ml of the mixture. The OD was measured, and the difference between the sample and blank OD values indicated the cellulase enzyme activity of the cellulolytic bacteria in degrading cellulose.

3. Results and Discussion

Cellulolytic bacteria isolated from the soil of sugarcane garden which is a layer of soil that is mixed with the litter of sugarcane plants rich in nutrient from the litter and the activity of organisms in the soil [23]. The sample soil is derived from 5 different points in the subdistrict of Berbah, Yogyakarta. Soil samples were then taken to the laboratory of microbiology, Fac of Biology UGM for isolation microbes from the sugarcane garden soil. Each sample point is selected five isolates (5 colony replications) so there are 25 samples of bacterial colonies obtained (Table 1).

Table 1. Bacterial isolation in Carboxy Methyl Cellulose medium

Sample replication number	Code of Isolates				
	I	II	III	IV	V
1	T1CI	T1CII	T1CIII	T1CIV	T1CV
2	T2CI	T2CII	T2CIII	T2CIV	T2CV
3	T3CI	T3CII	T3CIII	T3CIV	T3CV
4	T4CI	T4CII	T4CIII	T4CIV	T4CV
5	T5CI	T5CII	T5CIII	T5CIV	T5CV
Total	25				

*Note : + results for Congo Red assays

3.1. Qualitative Assays

On the test screening results apparently, there is not all isolate are suspected to be cellulolytic bacteria can degrade cellulose. From 25 isolates, only 3 isolates were able to degrade cellulose well on the CMC medium by showing positive results (clear zone) after being added to the Congo Red reagent and washed with NaCl. As well as Iodine tests, visible results of the clear zone around the bacterial colony after washing with aquadest. The following results indicate a positive isolate degrades cellulose.

(a) (b) (c)

Figure 2. Congo Red Test on Isolates (a) T2CIV, (b) T4CIV, (c) T2CI

In the Iodine assay the results are as follows:

(a) (b) (c)

Figure 3. Iodine Test on Isolates (a) T2CIV, (b) T4CIV, (c) T2CI

Based on Figures 2 and 3, it is known that all isolates show clear zones around the colonies of bacteria that grow with the Congo Red test and Iodine test. Critically, a negative halo does not necessarily mean that an isolate lacks cellulolytic potential. The Congo red–CMC assay preferentially detects endoglucanases acting on soluble/amorphous cellulose and may miss isolates that (i) express mainly exoglucanases (cellobiohydrolases) or cell-associated enzymes active on crystalline substrates (e.g., Avicel), (ii) produce β-glucosidase but have low endo-activity, or (iii) require induction (e.g., growth on cellulose or cellobiose) to secrete enzymes at detectable levels [12-13]. Incomplete hydrolysis can also arise from insufficient β-glucosidase activity, leading to cellobiose accumulation that inhibits upstream cellulases and limits glucose release, which in turn depresses growth and OD when cellulose is the sole carbon source.

3.2. Quantitative Assay

Based on DNS Assay, obtained result of optical density value and conversion result with standard curve as follows

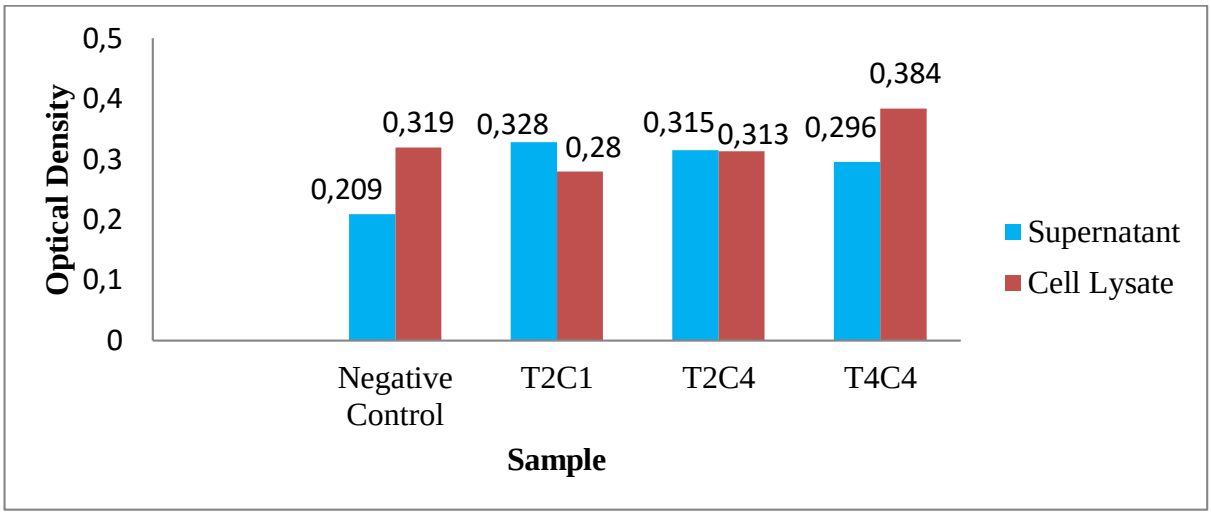

Figure 4. Optical Density Value of Three Isolates after DNS Assay using CMC

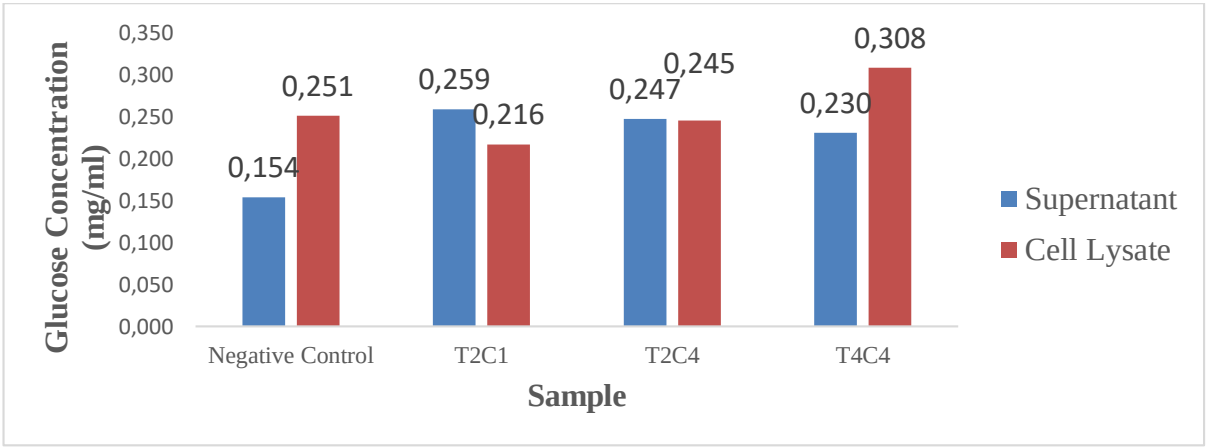

Figure 5. Graph of Conversion Value OD Spectrophotometer Results with Standard Curves

In the picture above there is a graph of the value of Optical Density (OD) using spectrophotometer by testing isolate T2CI, T2CIV, T4CIV and negative control, which is liquid medium without

treatment. In the the supernatant, the OD of all isolate are higher than the OD of negative control of supernatant. OD of negative control is 0.209, OD of T2C1 is 0.328, OD of T2CIV is 0.315, OD of TIVC4 is 0.296. While in the cells lysate, the OD of almost all isolate is lower than the value of the negative control cell lysate (0.319), the OD of TIIC1 is 0.28, and the OD of TIIC4 is 0.313. Only OD of TIVC4 has the higher number than negative control: 0.384. Once converted with a standard curve, the value pattern obtained is also similar.

Based on the results obtained in the qualitative assays of cellulase enzymes both in Congo Red assay and Iodine assay test it can be concluded that T2CIV, T4CIV, T2CI isolates are called cellulolytic bacteria because they are able to degrade cellulose in CMC media for growth [24]. The main function of cellulase enzymes is to make changes to cellulose around it so that the cellulose is converted into simple compounds, such as glucose which can be used as a source of carbohydrates in the metabolic processes of microorganisms.

Clear zones show hydrolytic activity by extracellular cellulase enzymes which are excreted by cellulolytic bacterial isolates. The hydrolysis products in the form of simple monosaccharide sugars and simple sugars do not form a bond with reagen Congo Red. Based on Anand et al., [25], Congo Red will bind specifically to polysaccharides that have β-1,4 glycoside bonds contained in CMC. While the red color shows the remaining cellulose that is not hydrolyzed so that the formation of cellulose is colored by Congo Red. Removing reagent with NaCl is used so that the clear zone formed can be clearly seen. Congo Red is a sodium salt of benzidinediazobis-1 naphthyl-amine-4 sulfonic acid ($C_{32}H_{22}N_6Na_2O_6S_2$) so that this dye will dissolve and be washed away by other salts (NaCl). Thus, the clear zone formed will become increasingly clear [26].

Cellulose as the most abundant compound on earth is composed of 8000-12000 units of glucose with β-1,4-glucoside. Bonds β-1,4-glucoside on cellulose fibers can be broken down into glucose monomers by cellulase, an enzyme consisting of three main types of enzymes: endoglucanase, eksoglucanase and β-glucosidase [27].

Cellulolytic microorganisms able to produce endoglucanase, exoglucanase and β-glucosidase which work synergistically in degrading cellulose [6]. Endo-1,4-β-glucanase enzyme breaks the cellulose chain randomly then exo-1,4-β-glucanase will remove glucose and cellobiose from the rest of the cellulose chain, and 1,4-β-glucosidase will hydrolyze cellobiose and other dissolved cellodextrine molecules become glucose molecules [27].

The long chain of cellulose contained in CMC media that is amorphous (irregular) is very easily broken down by cellulolytic bacteria, so the activity of the cellulase on the CMC substrate is an enzyme activity endo-1,4-β-glucanase acting on the chain in CMC to produce oligosaccharides or shorter cellulose chains [28]. Cellulose contained in CMC media on the media around the bacterial colonies will be used up so that when the coloring with Congo Red there is a bright zone because there is no bond between cellulose and Congo Red where cellulase enzyme is indicated to be produced by isolates so that cellulose is hydrolyzed, whereas in regions cellulose which will still bind with the Congo Red stain and the media remains red.

Quantitative measurement of cellulolytic activity is carried out by centrifuging isolates in the NB medium to separate the cell components (pellets) and the liquid (supernatant) used for examination. Then for supernatants added 1% CMC diluted in 10 mm Potassium phosphate buffer. The function of adding CMC is as a source of cellulose to be overhauled by cellulase enzymes produced by cellulolytic bacteria while potassium phosphate buffer as a material that can maintain the pH of the solution so as not to affect the structure and biological activity of enzymes (non-denatured enzymes). Following a 15 minute incubation period at 30 °C, DNS reagent was added, boiled for five minutes, and then cooled. A spectrophotometer set at 540 nm was used to measure the activity of cellulase enzymes. The aim is to incubate at 30° C so that the the temperature is the optimum for the enzyme to hydrolyze the substrate.

The addition of DNS functions as an oxidizing compound that can reduce reducing sugars as well as complex yellowish orange compounds that will color the solution so that it can be measured by uv vis spectrophotomers. The purpose of the analysis with the DNS method is to determine the activity of cellulase enzymes based on reducing sugar levels formed as the result of substrate hydrolysis by cellulase enzymes.

The DNS method was chosen in this study because it is a commonly used method for measuring cellulase enzyme activity by measuring the amount of reducing sugar formed and this method is also easy to do to measure large quantities of samples [29]. This DNS method is based on the reduction of 3.5 dinitrosalicylic acid to 3-amino-5-dinitrosalicylic acid. As long as there is reducing sugar in the test solution, the reaction will continue while the aldehyde group in the polysaccharide chain is oxidized to the carboxyl group and the aldehyde sugar group reduces dinitrosalicylic acid. The color change that occurs in the DNS reagent is from yellow to reddish orange.

According to the results, the OD value of all isolates is greater than the negative control OD value of Supernatan. This result indicated that cellulolytic bacteria produce a cellulase enzyme that is used to break down cellulose in the medium and produce glucose. As a result, the cellulase enzyme produced by cellulolytic bacteria is used to convert the medium's cellulose into glucose. These results also indicate that the cellulase enzyme produced by the bacteria we isolated is an extracellular cellulase enzyme, which is secreted by the cells outside their bodies.

In cell lysate, the OD value of isolates was lower than that of the negative control lysate cells: T2CI and T2CIV. The isolate OD value should exceed the negative control value. This can happen because bacteria are thought to not produce cellulase enzymes. According to Nurmayani et al., 2007 [30], this is possible in isolates that can produce cellulase enzymes, but the enzymes produced do not function optimally due to their low purity. It is possible that the enzyme still contains other components, such as inhibitors, that can interfere with its function. In addition, the decrease in the pellet OD value of isolates from the negative control can be caused by the isolate being unable to degrade cellulose completely. One of the stages of the cellulase enzyme is interrupted or does not produce β-glucosidase which plays an important role in breaking down the cellobiose chain into glucose [31]. The cellulase produced is an inductive enzyme whose biosynthesis is influenced by an inducer of cellulose found in CMC substrates. Cellulase works synergistically in the process of reshaping cellulose into glucose. The use of other types of cellulose carbon sources (besides CMC) can also be used to obtain more sensitive cellulase enzyme test results from cell lysate.

To strengthen interpretation and comparability, we recommend complementing OD with quantitative sugar release (e.g., DNS assay for reducing sugars) and a standard enzyme panel (CMCase, FPase, β-glucosidase) on multiple substrates (CMC for endoglucanase, Avicel or filter paper for exoglucanase, and cellobiose for β-glucosidase), as different assay substrates capture distinct facets of the cellulase system and often reveal activity missed by a single CMC/OD screen [13],[19],[29].

4. Conclusion

The conclusions of this research were there are three isolates with cellulolytic activity isolated from sugarcane fields in Berbah subdistrict, Sleman: T II C4, T II C1, dan T IV C4. Cellulolytic activity from those isolates shown clearly by qualitative methods using Congo Red and Iodine Assays. However, in the quantitative method using the DNS Assay, those isolates have positive activity only in supernatant samples and not in cell lysate samples, indicating that the cellulase enzymes produced by those isolates were extracellular enzymes.

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