Eksakta : Berkala Ilmiah Bidang MIPA
VOLUME 22 NO 01 2021, pp 27-34
ISSN : Print 1411-3724 — Online 2549-7464
DOI : https://doi.org/10.24036//eksakta/vol21-iss2/257

Eksakta
Berkala Ilmiah Bidang MIPA
http://www.eksakta.ppj.unp.ac.id/index.php/eksakta

Article

Isolation of Cellulolytic Microbes from Bio-Slurry

Article Info

Article history :

Received February 22, 2021
Revised March 16, 2021
Accepted March 18, 2021
Published March 26, 2021

Keywords :

Cellulolytic microbe, cellulase enzyme, bio-slurry, Carboxyl Methyl Cellulose (CMC)

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Abstract. Cellulolytic microbes produces cellulase enzymes that can degrade cellulose. Cellulolytic microbes are found in many habitats, including in cattle waste (bio-slurry), which is result of hydrolysis of cattle feed ingredients sourced from cellulose. Cellulase enzymes play a role in the process of hydrolyzing cellulose into glucose. This study aims to isolate cellulolytic microbes from bio-slurry and measure activity of cellulase enzymes in cellulolytic microbes isolated from bio-slurry. We are taking bio-slurry samples with 5 sample points and isolating the microbes in Carboxyl Methyl Cellulose (CMC) medium, cellulase enzyme activity qualitative test with Congo Red Assay, and perform quantitative test with the DNS Assay. Microbial isolates which shows positive results for cellulolytic activity in the qualitative test called SIIC1, SIIIC5, SIIC3. In the quantitative test can be seen based on Optical Density (OD) for cell lysate, SIIC1 0.298; SIIC3 0.186; SIIIC5 0.247; and a 0.332 for blank solution. Whereas supernatant obtain SIIC1 value of 0.237; SIIC3 0.212; SIIIC5 0.198; and 0.195 for blank solution. Based on the results, it can be concluded that the cellulolytic activity of microbes in bio-slurry based on OD values shows good results in supernatant, but it's not significant enough in the cell lysate.

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1. Introduction

Cattle is one of the ruminant animals which is quite easy to find. Ruminants can use high fiber ingredients that contain cellulose. In Cattle's rumen there is a process of decomposing cellulose into glucose [1]. Cattle's feces as the result of digestive process can be used as biogas and its pulp is mixed with soil called bio-slurry. Bio-slurry contains cellulose which is a straight chain polymeric carbohydrate (1,4)-ß-D glucose shaped like fiber, clay, insoluble in water, and is found in the cells

wall, especially in the stalk, stem, branch, and all woody part of plant tissue [2][3]. Those cellulose Bio-slurry had a lot of nutrition for a lot of plants such as Nitrogen (N), Phosphor (P), Kalium (K), Calcium (Ca), Magnesium (Mg), and Sulfur (S). Bio-slurry also contain micronutrient such as Iron (Fe), Mangan (Mn), Cuprum (Cu), and Zinc (Zn) [4].

Cellulose which was major compound of cell wall can be converted into simple carbohydrates by cellulolytic microbe [5][6][7]. Cellulolytic microbe is microbe which have ability to decompose cellulose into glucose and make it as a source of carbon. Cellulolytic microbe can synthesize cellulase enzyme if they growth on cellulose environment [8]. The benefit of cellulase enzyme which extracted from microbe is very broad. Beside in food and industry sector, the use of cellulase enzyme from microbe can provide solution for pollution problem by reducing amount of cellulose waste like leaf heap, agricultural waste, and can be processed into organic fertilizer [9][10].

In recent research, we know that these cellulolytic microbes widely found in several ecosystem such as in the forests, ruminant digestive animal tracts, until husbandry area [11]. As we know that bio-slury produced by ruminants' animal, which was abundance with cellulolytic microbes, there are gap information for research about microorganisms that found in bio-slurry in Indonesia, especially microorganisms that had cellulase activity.

The aim of this research was to study the presence of cellulolytic microbe from bio-slurry and know the level of enzyme activity produced by these microbes based on the value of Optical Density. This data will become a novel result for cellulolytic microbe research, especially using in bio-slurry.

2. Method

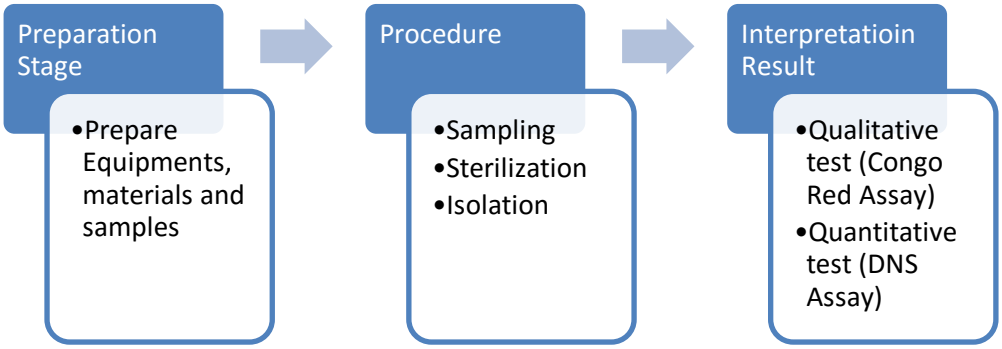


Figure 1. Research Flow Chart

2.1. Preparation Stage

This research performed in the Laboratory of Microbiology, Faculty of Biology, Universitas Gadjah Mada. Bio-slurry collected from The Center of Agrotechnology Universitas Gadjah Mada (PIAT UGM), Kalitirto, Berbah, Sleman. Next step, bio-slurry sample is processed in Microbiology Laboratory, Faculty of Biology UGM for further observation on cellulolytic microbe.

The equipments which used in this research are vortex, analytical scale, test tubes, petri dishes, test tubes rack, bunsen, inoculation loop, drygalski, incubator, mixer, LAF, autoclave, refrigerator, stove, pan, erlenmeyer, gas lighter, tube, pipette, label paper, ziplock, centrifuge, and spectrophotometer. The materials which used in this research are bio-slurry, distilled water, 70%

alcohol, CMC medium, LB medium, YPD medium, Congo red 1%, NaCl 1%, CMC 1%, potassium phosphate (P) buffer, DNS reagent, NB medium, cell lysate, glass beads, seal, paper cover, and cotton.

2.2. Procedure

In this research, sample took from PIAT UGM with 5 sampling points. Sample is taken from soil which has mixed with cow feces called bio-slurry. Sample which is taken earlier coded for first point called SI, second point was called SII, and so on until the fifth point. After bio-slurry is put into the ziplock then coded it according to sampling point. Bio-slurry then carried to laboratory to do further research about cellulolytic microbial activity. Then stored at room temperature in a cupboard.

All equipment had been sterilized with autoclave at 2 atm and 121°C for 3 hours. The test tube is filled with 9 ml aquadest by using a sterile pipette. One-gram bio-slurry was homogenized for a few moments with vortex in the first test tube. We perform serial dilution to another test tube for 6 times (10^{-1} until 10^{-6}), then homogenized again until well blended [12]. Final bio-slurry suspension was poured 1 ml had spread in a petri dish for isolation [13][14]. After that, 20 ml of CMC agar is poured into petri dish which contained microbe by using pour plate method. Petri dish is covered and shakes clockwise. The purpose is to make microbes spread well. Microbes was incubated at 37°C for about 3 days in an incubator [15].

2.3. Interpretation Results

Microbial isolates took from NB medium, then inoculated with point method in center of the petri dish contained CMC. After that, microbial isolates incubated at 37° C for 48 hours in an incubator. Colony in CMC medium was rinsed with water until microbial colony disappear. Cellulolytic activities in microbes can be observed by using Congo Red method. Congo Red solution is poured to form a thin layer. Then put on a shaker for 10 minutes. When it's done, remove the rest of Congo Red solution to waste disposal. After that, NaCl is poured into petri dish and put it on a shaker for 10 minutes. After NaCl is distributed well, the rest of it is removed to waste disposal. Petri dish is stashed in cupboard and left for an hour. The positive result identified if there is a clear zone [16].

Positive isolates from the Congo Red Assay test then tested quantitatively with DNS Assay method to see the optical density value and interpret numerically for the positive samples [17]. The first step, isolates are inoculated in tilted agar medium and stashed in cupboard, then left it for 1-2 days. After microbes grow, inoculated again in NB medium 4 ml with inoculation loop. Then sample was incubated for one day in an incubator. CMC 1% is added 400 µl into 10 mm potassium P buffer then centrifuged until supernatant and pellets are separated. The centrifuge process did 3 times for 2 minutes. Supernatant was taken 100 µl from each sample and mixed with CMC medium. After that, it incubated for 30 minutes at 45° C. DNS reagent 500 µl is added to microbial isolate 500 µl so the total is 1000 µl (1 ml). Tube was sealed with plastic seal tightly and boiled for 5 minutes at 100° C. After that, tube cooled for a while at room temperature. Optical density value observed by using spectrophotometer with a wavelength 540 nm. Blank solution made as negative control consisted of NB medium 100 µl and CMC in potassium P buffer 400 µl. Pellet lysed by adding 0.1 ml of glass beads to tube. After that, 200 µl of buffer lysis is inserted into tube and homogenized it. The way to get cell lysate is centrifuge an isolate again for 2 minutes. After supernatant and pellet have separated, only 100 µl supernatant is taken then mixed with 400 µl CMC. Optical Density value can be seen on spectrophotometer. A blank solution for pellet is made from 100 µl lysis buffer plus 400 µl CMC in potassium P buffer. The ability of cellulolytic microbe to degrade cellulose can be seen from the difference in value between blank solution and sample.

3. Results and Discussion

3.1. Results

Based on research that has been done, data which obtained like tables containing isolate codes, figure for qualitative test, and graph for quantitative test. These data can be seen as follows.

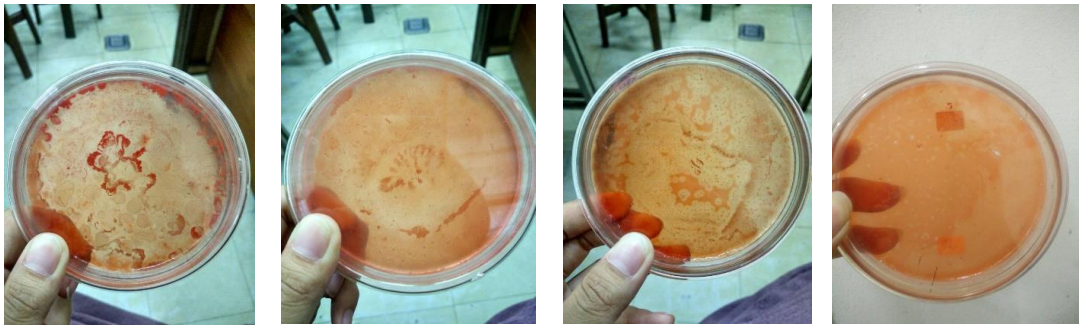
Table 1. Cellulolytic Microbe Isolates in CMC medium

Sample Number	Microbial Isolates Code at 5 Sampling Points				
	CMC				
	I	II	III	IV	V
1	SIC1	SIIC1	SIIIC1	SIVC1	SVC1
2	SIC2	SIIC2	SIIIC2	SIVC2	SVC2
3	SIC3	SIIC3	SIIIC3	SIVC3	SVC3
4	SIC4	SIIC4	SIIIC4	SIVC4	SVC4
5	SIC5	SIIC5	SIIIC5	SIVC5	SVC5
Total			25		

*Noted : Positive results for cellulase assays

Qualitative Test Cellulase Enzyme Activity

In this research, Carboxyl Methyl Cellulose substrate degraded by cellulase enzyme into glucose [18]. Congo Red Assay was used in the qualitative method testing to check cellulase enzyme activity. Congo Red assay did by selecting an isolate with good growth, then dripped with 1% Congo Red solution. After that, washed with 0.1 M NaCl solution. Cellulase enzyme activity is characterized by formation of clear zone on microbial growth medium. The following result shows positive isolate in degrading cellulose.



a **b** **c** **d**

Figure 2. Congo Red Assay on Isolates (a) SIIIC5, (b) SIIC3, (c) SIIC1, (d) negative control

Quantitative Test Cellulase Enzyme Activity

Based on cellulase enzyme activity test, the result of optical density value was shown as follows:

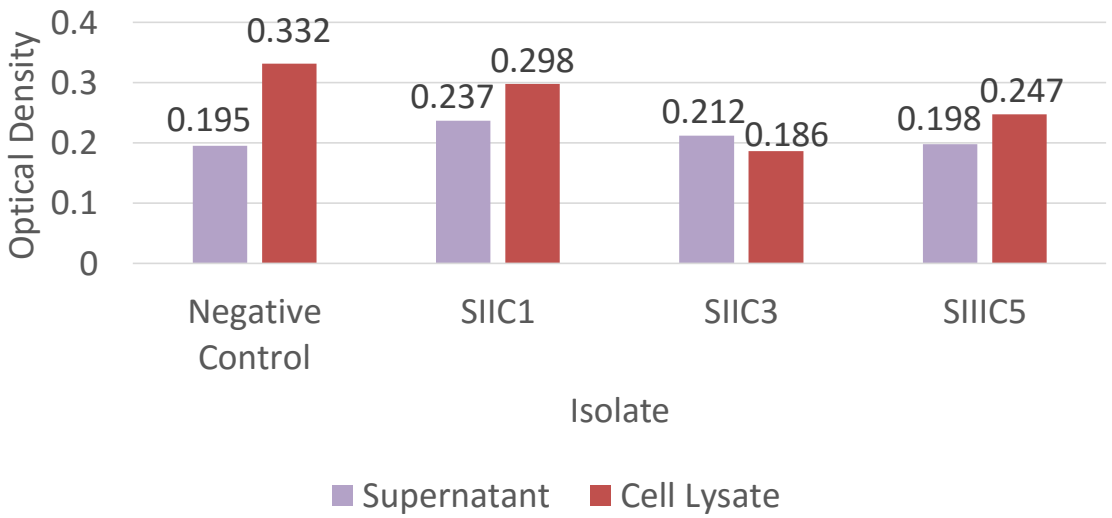


Figure 3. Graph of Optical Density Value with Spectrophotometer

In the figure above, there is a graphic of optical density value tested by spectrophotometer [19]. The isolates are SIIC1, SIIC3, SIIIC5, and blank solution which is liquid medium without treatment as a negative control. The supernatant shows OD value of all isolate are higher than OD value of blank solution that is 0.195. The cell lysate shows OD value of all isolate are lower than OD value of blank solution that is 0.332.

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3.2. Discussion

The ability of microbe to produce clear zone on cellulolytic specific media indicates that microbe can produce cellulase extracellular enzymes which secreted by cellulolytic microbial isolates [20][21]. That hydrolysis product present as a simple monosaccharide sugar and it does not form a bond with Congo red. Congo red specifically binds polysaccharides with β-1,4 glycoside bond found in CMC medium [22]. Cellulose which is not hydrolyzed will be colored by Congo red. The purpose for washing isolate with NaCl is to see a clear zone clearly.

According to Zverlova et al. [23], Congo red ($C_{32}H_{22}N_6Na_2O_6S_2$) is a sodium salt which washed out easily by other sodium salt, such as NaCl. A clear zone can be observed well. The bigger clear zone produced mean the greater ability of microbe to produce cellulase enzyme. Clear zone can be seen because of CMC hydrolysis found in cellulolytic microbial growth medium. Negative control used as a comparison to determine whether isolate was positive or negative. Negative control made from CMC medium which had been given Congo red and then washed with NaCl but didn't contain cellulolytic microbe.

Cellulolytic microbes can produce endo-1,4-β-glucanase, ekso-1,4-β-glucanase and 1,4-β-glucosidase which degrade cellulose [24][25]. According to Fikrinda [26] the function of enzyme endo-1,4-β-glucanase, ekso-1,4-β-glucanase, and 1,4-β -glucosidase in sequence are to break cellulose chain randomly, glucose or even cellobiose will be removed from rest of the cellulose chain which is not degraded, cellobiose and other cellodextrin molecules will be hydrolyzed into glucose molecule. CMC medium contains cellulose which will be used up by microbes in it. When Congo red staining is done, it will be appearing a clear zone. The clear zone is formed due to absence of a bond between cellulose and Congo red because cellulose has been hydrolyzed.

In Figure 1, three isolates showed positive result because they produced a clear zone. From three isolates, SIIC1 has a clearest clear zone. This proves that SIIC1 has the strongest ability to degrade cellulose compared to 2 other isolates. Measurement of cellulase enzyme activity was done by adding supernatant and cell lysate to CMC solution in potassium P buffer. CMC has a function as a source of cellulose which is hydrolyzed by cellulase enzyme.

Addition of phosphate buffer has a function as a material that maintains pH solution and enzyme which is not denatured. Next, the solution mixture incubated for 30 minutes at 45° C. The incubation is carried out at 45° C to optimizing hydrolysis process and this temperature was optimum temperature of enzyme to hydrolyze CMC substrate. After that, DNS reagent added into tube and sealed with plastic seal tightly. The DNS reagent has a function as an oxidizer compound which reduces sugar. It also gives a yellowish orange color to solution so it can be measured with spectrophotometer. Samples and negative control was boiled for 5 minutes at 100° C and cooled for a while before measured with spectrophotometer.

Measurement of enzyme activity was done by determining concentration of reducing sugar with making a glucose standard curve. Glucose solution is chosen as a solution for making standard curve because glucose is one of reducing sugar produced from substrate hydrolysis by cellulase enzyme. Enzyme activity can describe a purity of an enzyme. The higher an enzyme activity, the purer an enzyme is. The enzyme activity results will be same as reducing sugar results pattern.

Figure 2 shows results for qualitative test of cellulase enzyme activity. The three isolates compared with negative control both in supernatant and cell lysate. In supernatant sample, OD values of each isolate were higher than OD values of blank solution. It indicates that there is cellulase enzyme produced by cellulolytic microbes. It used to remodel cellulose contained in medium and it converted to glucose.

In cell lysate sample, a value of blank solution is the highest compared to three isolates. There is an error in cell lysate sample because value of blank solution should be lower than isolates OD value. It should be higher than blank solution value. This condition can be occurred because it is suspected that cellulolytic microbe did not produce cellulase enzyme, or cellulase enzyme that produced can not degrade cellulose well enough. Another possibility is an enzyme which produced by cellulolytic microbes are not optimal. It occurs due to low enzyme purity. This happen because an enzyme may still contain other components and enzyme's performance can be hampered [27].

4. Conclusion

This research proves that there are cellulolytic microbes activity in isolates from bio-slurry at PIAT UGM. The microbial isolates which show positive result for cellulolytic activity are SIIC1, SIIIC5, and SIIC3. The ability of cellulolytic microbes isolated from bio-slurry based on optical density value shows good result for supernatant samples but not for cell lysate samples.

Acknowledgment

The research was conducted using 24th-Science and Technology Research fund from ITSF Foundation, Young Lecturer fund UGM 2019 and Hibah Kolaborasi Dosen dan Mahasiswa Faculty of Biology UGM 2020. I also want to thank Mr. Sumarno for his assistance in Microbiology Laboratory.

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