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Isolation and Characterization of Potential Lignocellulosic Degrading Bacteria from Chicken Manure Compost

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ABSTRACT

Lignocellulose is the main component that can be found in plants. Lignocellulose consists of three components, namely hemicellulose, lignin, and cellulose. The maximum utilization of lignocellulose cannot be carried out without being degraded, however there are difficulties in carrying out the degradation. The difficulty faced in the degradation process is the presence of lignin components that provide strength and stiffness to the plant, which make it is quite resistant to the degradation process. Lignocellulose degradation requires delignification. The delignification process can be facilitated by enzymes produced by a number of microorganisms including bacteria can produce lignocellulosic enzymes. This study aims to isolate and characterize lignocellulose-degrading bacteria in chicken manure, and to assess the ability of bacteria to degrade lignocellulose. The results obtained from this research showed that an isolate which has high similarity with Shigella flexneri bacteria which was found in chicken manure and exhibits a potential to degrade lignocellulose.

***Keywords**: Shigella flexneri; lignocellulosic bacteria; chicken manure; lignocellulosic enzyme.*

ABSTRAK

Lignoselulosa merupakan komponen utama yang dapat ditemukan pada tumbuhan. Lignoselulosa terdiri dari tiga komponen yaitu, selulosa, hemiselulosa, dan lignin. Pemanfaatan lignoselulosa secara maksimal tidak dapat dilakukan tanpa didegradasi, namun terdapat kesulitan dalam melakukan degradasi tersebut. Kesulitan yang dihadapi dalam proses degradasi adalah adanya komponen lignin yang memberikan kekuatan dan kekakuan pada tanaman sehingga cukup tahan terhadap proses degradasi. Degradasi memerlukan delignifikasi untuk menghancurkan komponen lignin. Proses delignifikasi dapat difasilitasi oleh enzim yang dihasilkan oleh sejumlah mikroorganisme, termasuk bakteri yang dapat menghasilkan enzim lignoselulase. Penelitian ini bertujuan untuk mengisolasi dan mengkarakterisasi bakteri pendegradasi lignoselulosa pada kotoran ayam, serta mengkaji kemampuan bakteri tersebut dalam mendegradasi lignoselulosa. Hasil yang diperoleh dari penelitian ini menunjukkan bahwa isolat yang memiliki kemiripan tinggi dengan bakteri Shigella flexneri yang terdapat pada kotoran ayam dan berpotensi untuk mendegradasi lignoselulosa.

***Kata Kunci**: Shiella flexneri; bakteri lignoselulotik; pupuk kandang ayam; enzim lignoselulase.*

BACKGROUND

Lignocellulose is one of the main components of plants. Lignocellulose consists of three components, that is hemicellulose, lignin, and cellulose (Imsya *et al.*, 2014). Lignocellulosic materials are abound in water hyacinth (Zulfikar *et al.*, 2020). This weed, has negative a impact due to their rapid growth in Lake Tondano, Minahasa Regency. Various measures have been done in tackling the growth of water hyacinth (Moningkey *et al*., 2021). One of the best approaches to control water hyacinth is to explore its potential utilization (Rezania *et al*., 2015).

Agricultural waste containing lignocellulose can be used to produce sustainable organic products, such as producing xylitol from xylose contained in hemicellulose chains (Purnawan *et al*., 2021), producing microcrystalline cellulose as a water-resistant bioplastic material (Nur *et al*., 2020), and produces phenol, benzene, toluene, xylene, carbon fiber, activated carbon and other composite materials as materials for energy sources (Rahayu *et al*., 2019).

The maximum utilization of lignocellulose can not be carried out without being degraded. However there are obstacle in carrying out the degradation. The difficulty faced in degradation process is the presence of lignin components that provide strength and stiffness to plants so that they are quite resistant to degradation process (Gonzalo *et al*., 2016). Lignocellulose degradation requires delignification (Einhuber *et al*., 2013). Delignification is a preprocessing that is useful for breaking lignin bonds and reducing the degree of polymerization and the crystalline nature of cellulose, so it can accelerate the degradation process of lignocellulose (Nurika *et al*., 2021). In this research will discuss biological delignification using microbes.

Delignification can be carried out biologically by bacteria or fungi by utilizing their produced enzyme. Bacteria can produce lignocellulase enzymes such as cellulase, laccase, and lignin peroxidase that can degrade lignocellulose (Murtiyaningsih and Hazmi, 2017; Rupaedah *et al*., 2019). Biological pretreatment has many advantages, namely low cost, low energy consumption, no use of chemicals, little negative impact on the environment, and no production of inhibitors. This is different from the physical, chemical, or physiochemical pretreatment which requires high costs, high energy consumption, and excessive use of chemicals that can damage the environment (Menon and Rao, 2012; Brodeur *et al*., 2011).

Bacteria can grow anywhere, including in chicken manure. According to Ma *et al*. (2020), some lignocellulosic degrading bacteria can be found in livestock manure, especially chicken manure which has cellulase enzyme activity. Nurliana *et al*. (2019), reported that cellulase enzyme found in chicken digestive tract. Based on the described background, this study focuses on isolation and characterization of lignocellulosic bacteria in chicken manure compost.

MATERIALS AND METHOD

This research was done at the Pharmaceutical Microbiology Laboratory, Faculty Mathematics and Natural Science, Sam Ratulangi University, Manado. The research period was starts from May to July 2022. The equipment used in this research included sterilizers, glassware, hotplate, magnetic stirrer, pH meter, petri dish, microscope, object glass, ose needle, spreader, incubator, pipette, micropipette, and Bunsen burner. The materials that were used in this research included chicken manure compost that has been processed purely without husks, Nutrient Agar (NA), CMC, Tannic Acid, $MgSO_4.7H_2O$, Agar, $(NH_4)2SO_4$, KH_2PO_4, H_2O_2, Tryptone Broth, Simmon Citrat Agar, Triple Sugar Iron Agar, Reagen Kovac, and ddH_2O.

The media used was Nutrient Agar (NA) media. Preparation of NA media was done according to the method of Napitupulu *et al*. (2019) which has been modified. NA media was put into a beaker glass of as much as 28 grams and 1L of distilled water was added. Then, the beaker filled with the media was

homogenized on a hotplate with a magnetic stirrer until all the ingredients were dissolved and boiled. When the material has dissolved and boils, it was put into an Erlenmeyer flask to be sterilized using an autoclave at 121^{o}C for 30 minutes with a pressure of 15 psi. The media that had been sterilized was then poured into a 20 ml petri dish, if it was not going to be used, then the media was stored in the refrigerator.

Isolation of bacteria was carried out with tools and materials previously sterilized and using the method of Kasi *et al.* (2020) which has been modified. The sample was diluted with a dilution of 10^{-1} to 10^{-5} and 0.1 ml was inoculated into the NA medium. Isolation of bacteria was done using the spread plate method. Then the bacteria were incubated at 35^{o}C for 24 hours. Bacteria that grew after incubation were purified on a new medium.

After being purified, the bacteria were tested for lignocellulosic enzymes. Lignocellulosic enzyme activity test was done using the method of Wahyuningsih and Zulaika (2018) which has been modified. Selective media was prepared with a composition of 2 g of peptone; 0.4 g dipotassium phosphate (K_2HPO_4); 3 g agar; 0.48 g ammonium sulfate ($(NH_4)_2SO_4$); 0.06 g magnesium sulfate ($MgSO_4.7H_2O$) with the addition of 1 g of CMC and tannic acid, then dissolved with 200 ml of distilled water. The medium that has been diluted with distilled water is sterilized in an autoclave for 30 minutes and after being sterilized, it was poured into a petri dish and then 1 oze of a colony is inoculated into the medium. The medium that had been inoculated with bacterium is incubated at 35^{0}C for 72 hours. The growing bacterium indicated that the bacterium could degrade lignocellulose. Bacterium that have grown on lignocellulosic media were sent to PT. Genetika Science Indonesia to be identified molecularly

Then the lignocelullosic sample was characterized. Observations were made macroscopically and microscopically. Macroscopic observations were made by observing the shape, elevation, surface, color of the colony, and gram staining. Microscopic observation of bacteria was carried out by observing the shape and arrangement of bacterial cells by gram staining, motility test, and biochemical tests according to standard methods. The biochemical tests carried out included catalase test, indole test, citrate test, and TSIA test.

RESULT AND DISCUSSION

Bacterial Isolation

Isolation of bacteria in chicken manure was done using the spread plate method with dilution from 10^{-1} to 10^{-5} (**Figure 1**) so that eight different bacterial isolates were obtained after being incubated for 24 hours at 35^{o}C. The isolated bacteria were coded according to macroscopic characteristics the eight isolates were designated as BESC, BESR, BILR1, BILR2, BIMR, ILLR, IRLR, and ILMC. The eight isolates that were characterized macroscopically were then purified. Purification according to Handoko *et al*. (2020) was an important step to obtain separate colonies called pure cultures from previously mixed bacterial cultures.

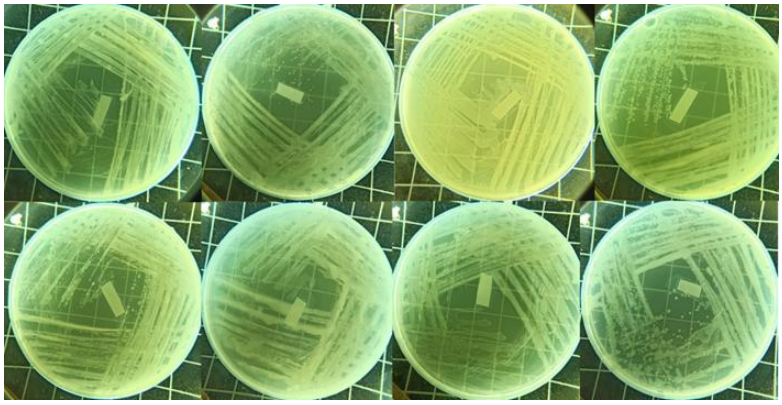


Figure 1. Pure isolates from chicken manure compost (top from left to right: BESC, BESR, BILR1, and BILR2; bottom from left to right: BIMR, ILLR, ILMC, IRLR)

Lignocellulosic Enzyme Activity Test

Lignocellulosic enzyme test was done by growing bacterial isolates on lignocellulosic media, if the bacteria grows then the bacteria have enzymes to degrade lignocellulose in it. The eight bacteria were isolated on lignocellulosic selective media, but only 1 bacterium could grow on lignocellulosic media, namely BESC bacterium (**Figure 2**). This bacterium were then purified and sent for molecular identification to determine the species of BESC bacterium.

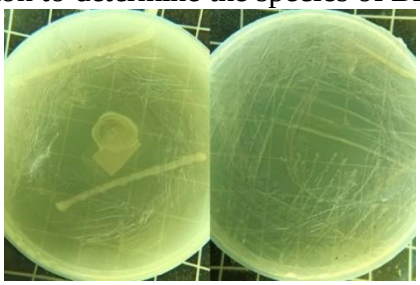


Figure 2. BESC bacterium on lignocellulosic media

Bacterium Characterization

BESC bacterium has macroscopic characteristic such as round shape with flat edges, small in size, convex elevation, and negative-gram (**Figure 3**). Gram staining according to Putri and Kusdiyantini (2018) is a very important staining method that aims to distinguish positive-gram and negative-gram bacteria. According to Leboffe and Pierce (2010), positive-gram bacteria will still have a purple dye from crystal violet after washing with alcohol, while negative-gram bacteria will not be able to retain the dye so that when stained with safranin, negative-gram will turn red. This happens because positive-gram bacteria have very thick peptidoglycan cell walls so when washed with alcohol, the cell walls narrow and they retain crystal violet dye. While microscopic characteristic of BESC isolate such as rod-shaped cells (**Figure 3**). According to Boleng (2015), there are 3 general shapes of bacterial cells namely, round (*coccus*), rod-shaped (*bacillus*), and spiral-shaped.

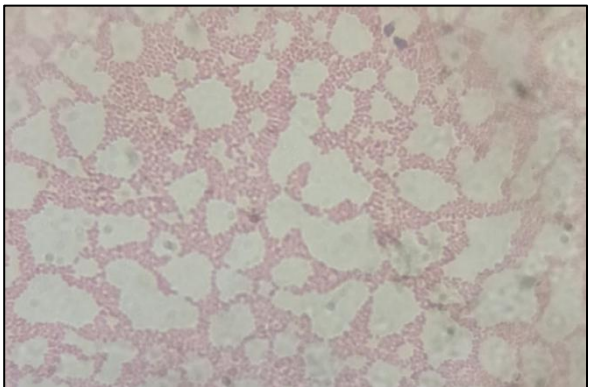
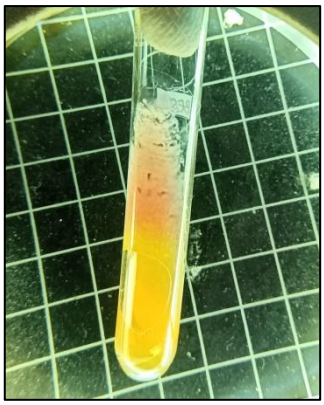
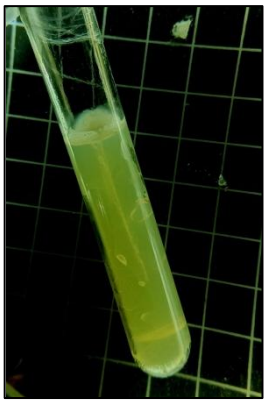


Figure 3. Bacterium cell shape after gram-staining

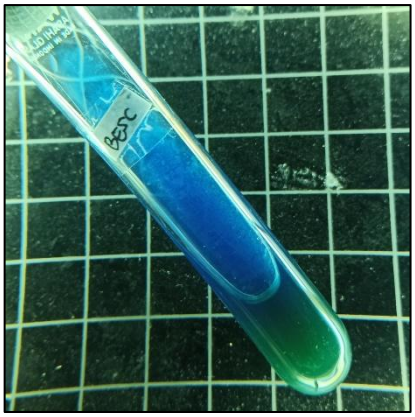


a. TSIA test



b. Motility test

c. Catalase test



d. Citrate test

Figure 4. Biochemical test (a. TSIA test showed positive result for sugar fementation and gas formation test; b. Motility test showed negative result; c.Catalase test showed positive result; d. Citrate test showed positive result.

Biochemical tests on this bacterium showed positive citrate test that means this bacterium can use citrat as carbon source, showed positive catalase test means has catalase enzyme, showed positive indole test means has tryptophanase enzyme, and showed negative motility test (**Figure 4**). Tests on TSIA media can prove that there are 4 tests, namely glucose test, sucrose and lactose test, gas formation test, and H_2S test. In the glucose test, the BESC isolate showed positive result, which means that the isolated bacterium could ferment sugar, while the

negative results were obtained from sucrose and lactose. According to Aini (2018), if the top of the TSIA media is red, the bacteria can ferment glucose, if the top and bottom are yellow, the bacteria can ferment sucrose and lactose, but if the top and bottom of the media are red, the bacteria cannot ferment all carbohydrates. The result for the H_2S test on the BESC isolate showed negative result, or at the bottom of the media, no black color was formed. The gas formation test showed positive results on the BESC isolate.

Molecular Identification

Based on the results of the analysis sent to PT. Genetika Science Indonesia using the 16s rRNA method and searched for DNA sequences using BLAST, the length of the PCR product was 1500kb (**Figure 5**). From the result of the analysis, it was found that the BESC bacterium isolate has the highest similarity with *Shigella flexneri* species, where the percentage identity between the DNA input and the DNA target was 99.79 % which indicated that the sequence of the DNA input was in accordance with the DNA target. However, the obtained data also showed that the BESC bacterium isolate has the similarity with *Escherichia coli* species with an identity percentage of 99.71 %. According to Stackerbrandt and Goebel (1994), samples of microorganisms using 16s rRNA markers are said to be identical at the species level if the percentage identity value is above 97.5 %.

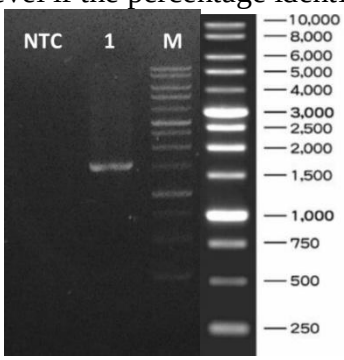


Figure 5. PCR Product showing the expected size around 1500kb

Shigella flexneri and *Escherichia coli* are closely related microorganisms as reported by Zuo *et al.* (2012). They also reported that several molecular studies placed *Shigella* within the *E. coli* species, but their research using the CVTree approach showed that four species of *Shigella* differed from *E. coli* and formed only sister species in the genus *Escerhicia*, the four species being *S. boydil*, *S. sonnei*, *S. flexneri*, and *S. dysenteriae*. **Figure 6** presents the BLAST result, where the *S. flexneri* and *E. coli* look similar even with their sequencing DNA.

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Shigella flexneri strain WW4-5 16S ribosomal RNA gene, partial sequence	2569	2569	99%	0.0	99.79%	MW832279.1
Escherichia coli strain BE42 16S ribosomal RNA gene, partial sequence	2566	2566	100%	0.0	99.71%	EF560785.1
Escherichia coli strain SA10 16S ribosomal RNA gene, partial sequence	2562	2562	100%	0.0	99.64%	MT535596.1
Shigella flexneri strain WW1-1 16S ribosomal RNA gene, partial sequence	2562	2562	99%	0.0	99.71%	MW832245.1
Escherichia coli strain EC20017429 chromosome, complete genome	2560	17835	100%	0.0	99.64%	CP071711.1
Escherichia coli strain Z0117EC0116 chromosome, complete genome	2560	17779	100%	0.0	99.64%	CP098192.1
Escherichia coli strain MEI005 chromosome, complete genome	2560	17752	100%	0.0	99.64%	CP071259.1
Escherichia coli isolate 410 genome assembly, chromosome: main	2560	17912	100%	0.0	99.64%	OW967802.1
Escherichia coli isolate 23 genome assembly, chromosome: main	2560	17835	100%	0.0	99.64%	OW848980.1
Escherichia coli strain SH21PTE31 chromosome, complete genome	2560	17835	100%	0.0	99.64%	CP097181.1

Figure 6. BLAST Result

In this research, the BESC isolate code was closer to the characteristics of *Shigella flexneri*, as reported by Wang *et al.* (2011), the characterization of *S. flexneri* was rod-shaped, gram-negative, non-motile, and able to ferment glucose, as compared with the characterization of *E.coli* from the research of Ummamie *et al.* (2017) have the characteristics, that is rod-shaped cells, gram-negative, motile, and can ferment glucose. In the biochemical test, BESC has non-motile properties, this makes BESC close to the characteristic of *S. flexneri*. According to Wang *et al.* (2011), *S. flexneri* has the ability to degrade and saccharify cellulose because it has cellulase enzymes. This is also confirmed by research from Disale and Dixit (2020) which showed that *S. flexneri* species has the ability to degrade lignocellulosic biomass because it has enzyme activity such as cellulase, xylanase, and laccase.

CONCLUSION

Lignocellulosic degrading bacterium are found in chicken manure compost and has macroscopic characteristics such as round shape with a flat edge, small in size, convex elevation, and negative-gram, while microscopic characteristics such as rod-shaped cell, and biochemical tests on this bacterium showed positive citrate test, positive catalase test, positive indole test, positive sugar fermentation test, positive gas formation test, and negative motility test. After this bacterium characterized, then the bacterium identified molecularly and got the result that *Shigella flexneri* has similar with BESC bacterium with percentage identity of 99.79%, and the characteristic show similarities. Further research is needed to determine the quantitative lignocellulosic enzyme activity of *Shigella flexneri* bacterium in chicken manure compost.

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