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Screening and Isolation of Cellulolytic Bacteria from Gut of Black Soldier Flays Larvae (*Hermetia illucens*) Feeding with Rice Straw

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Abstract

A research on screening and isolation of cellulolytic bacteria from the gut of larvae Black Soldier treated rice straw feed has been conducted. The purpose of this study is to get the type of cellulolytic bacteria from the gut of larvae and bacteria that have the highest potential to degrade cellulose. Screening and isolation method applied by using intestinal larvae obtained from larval gut vortex at a speed of 1500 rpm. Furthermore, dilution graded from 1 to 10 and grown in media CMC (carboxyl methyl cellulose) at 37 ^{0}C and incubated for 48 hours. Observations were made based on the characteristics of the microscopic, macroscopic, biochemical test, cellulolytic activity and the activity of cellulase enzymes selected bacteria. The results showed a 9 cellulolytic bacteria from the gut of the larvae. *Bacillus* sp. is a bacteria that have the highest potential with cellulolitic activity 2.1 mm (dz/dk), the exponential phase of hour at the 24th, and cellulase enzyme activity of 0.4 U/mL at pH 7 and 0.41 U/mL at pH 8.This research showed that the Black Soldier Flays Larvae (*Hermetia illucens*) have competencein organic waste degradation, because in Black Soldier Flays *Larvae's* gut, cellulolitic enzyme is produced by cellulolitic bacteria, specially *Bacillus* sp,

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INTRODUCTION

Rice straw as organic waste has a chemical composition (in dry weight) about32.1% cellulose, 24% hemicellulose and 18% lignin (Howard et al., 2003) Results of other studies Wannapeera et al. (2008) showed that rice straw containing 35.7% hemicellulose, 32% cellulose, lignin 22.3%, and 10.1% ash. The crude protein of rice straw is around 2-7 (Drake et al., 2002). Research conducted by (Hong et al., 2007) states that on the thermophilic phase of rice straw, there was lignocellulose degrading microbes such as *Bacillus* sp. groups. Feeding hay to the larvae allows the bacteria non-normal intestinal bacteria come into the intestine Black Soldier fly larvae. Larvae *Black Soldier* has been found to contain *Bacillus subtilis* microbes on the skin and intestines (Yu et al., 2011).

B. subtilis is a bacterium that produces the enzyme α-amylase, which is able to hydrolyze α-1,4 bond-glycosidic polysaccharides (starch) into smaller molecules, even to convert starch into simple sugars (Demirkan, 2011). *B. subtilis* also produce a protease enzyme, which catalyzes the termination of peptide bonds in proteins. In addition to protease, *B. subtilis* produce lipase enzymes that function down fats into fatty acids and glycerol (Pouderoyen et al., 2001; Ma et al., 2006; Singh et al., 2010).

Another important enzyme which is also known produced by *B. subtilis* is a cellulose (Shaheb et al,.2010); (Yin et al., 2010). Cellulose enzyme in the bacteria *B. subtilis* is Carboxymethyl cellulose (CMCase), β-glucosidase, Avicelase, and xylanase (Kim et al., 2012). Therefore, *B. subtilis* can be categorized as cellulolytic bacteria. Cellulolytic bacteria are bacteria capable of degrading and utilize cellulose as a source of carbon and energy (Baharuddin et al., 2010). The working nature of enzymes is influenced by several factors, including the temperature and pH. Generally *Bacillus* is a bacteria that can grow in the spacious range of temperature and pH, and relatively easy to be isolated from a wide variety of environments, and able to grow in synthetic media (Johnvesly & Naik, 2001).

The benefits of this research is, it is known that the larvae of the black soldier has the potential to be used to degrade organic waste in the gut because the larvae are cellulolytic bacteria that aids in the hydrolysis of cellulose. Besides cellulolytic bacteria that can be isolated can be used as a biocontrol agent for composting cellulose produced by cellulolytic bacteria.

METHODS

Black Soldier larvae that have fed hay for 30 days, performed the surgery on the abdomen. A total of 1 gram of intestinal larvae were taken and then put into a test tube containing 0.9% NaCl physiological and mixed with vortex (retailing $^{-10}$), further dilutions terraced up 10^{-10} by taking 1 ml of the previous dilution and then diluted with 9 ml sterile NaCl, Cappuccino &Sherman, 1987). 1 ml of suspension taken from the larval gut dilution 10^{-3} to 10^{-10}, grown in a medium jelly containing 1 g of CMC; $MgSO_4$ 0.06 g, 0.225 g KNO_3, K_2HPO_4 0.25 g, 0.06 g $FeSO_4$, 0.012 g $CaCl_2$, 0.6 g yeast extract, 0.3 g of glucose, and jelly 6 g yeast extract. All the ingredients are mixed with aquadest of 300 ml, incubated for 24-48 hours at a temperature of 37 ^{0}C.Then the medium is sterilized by autoclaving at 121^{0}C for 15 minutes and a pressure of 1 atm.

Macroscopic observation referring to the guidelines Bergey's Manual of Determinative Bacteriology 9th, Microbiology A Laboratory Manual (Cappuccino & Sherman, 1987), *Pharmaceutical Microbiology* (Denyer et al., 2004), Analysis of Microbial Laboratory (Lay, 1994).The observed characteristics include the shape of the colony, the colony surface/elevation, the edge of the colony, and the color of the colony, whereas microscopic observations include cell shape, gram stain, catalase test, and test of endospores. Furthermore, the selection of the most potential bacteria produce cellulase enzymes. The observation of selected isolates are approaching on the characteristics of *Bacillus subtilis* bacteria.

Bacterial isolates were grown in Nutrient Broth media, then incubated in a *shaking incubator* at 120 rpm for 24 hours at a temperature of 35 ^{0}C. A total of 25 ml inoculum was inserted to 250 ml medium of cellulose enzymes production (PES) and incubated with a speed of 1500 rpm at a temperature of 35 ^{0}C for 48 hours (liquid culture), then the suspension was stored at 4^{0}C. Cellulase enzyme extract was obtained by centrifuging 10 ml of liquid culture with a speed of 4,000 rpm at 4 ^{0}C for 15 minutes.

A total of 1 ml of crude extract enzyme is added by 1% solution of CMC at various pH 5, 6, 7, and 8, incubated in *water bath* with a temperature of 40 ^{0}C for 10 minutes. To stop the enzyme activity, added DNS, 1.5 ml, and heated as long as 5 minutes, the enzyme activity was measured using a spectrophotometer at a wavelength of 540 nm (Miller, 1959).

RESULT AND DISCUSSION

From the observation of cultivation *Black Soldier fly* larvae, it was obtained the size and weight of larvae for 30 days showed an increase in the size and weight of larvae (Figure 1),

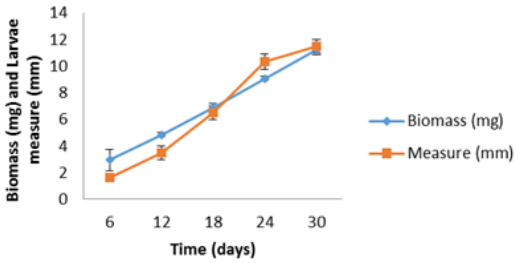


Figure 1. Correlation between larvae age and its biomass and length

Figure 1 is a graph of the increase larval biomass and the increase length of the larvae. The larval weight by age 6, 12, 18, 24, and 30 days in a row is 2.95; 4.85; 6.88; and 11.2 (mg). Whereas the length of the larvae in consecutive namely 1.55; 3.5; 6.5; 10; and 11.5 (mm). Black Soldier fly larvae to adults generally measure up to 20 mm (Sheppard, 1994). Larvae at the age of 30 days was then used for the isolation of bacteria from the gut. Results of macroscopic observation cellulolytic bacteria in CMC media are presented in Tables 1 and 2.

The results in Table 1 and 2, states that there are two isolates that indicated by Gram-negative and 7 isolates by Gram-positive. Cell wall of Gram negative bacterial contains less peptidoglycan than Gram-positive bacteria. This peptidoglycan have peptide cross links between glycans units and peptides that are less flexible than Gram-positive bacteria. Gram-negative bacteria is containing higher lipid and thinner cell walls that causing lipid solubility by the alcohol solution (Pelczar et al., 1998).

Catalase positive test results, showing that the bacterial isolates (1,3,4,5,6,7,8 and 9) are aerobic. Catalase enzyme produced by several types of bacteria in order to prevent oxidation of free radicals that can damage the bacteria. The enzyme catalase causes hydrolysis of H_2O_2 into H_2O and O_2 (Benson, 2002). Endospores positive observation is marked with a red vegetative cells and endospores negative marked with green (Lay, 1994). *B. subtilis* produces endospores (1.5 µm - 1.8 m) in oval or round shape and resistant to unfavorable conditions. Endospores is much more resistant to bad external influences than ordinary bacteria, the bacteria in the form of a vegetative state.

Table 3, based on positive test results on glucose, mannitol maltose, sucrose and lactose arabinose showing that the bacterium has an substrate enzyme breaker. These chemical compounds are used by microorganisms as an additional energy source (Lay, 1994). Motility test is performed to determine that the bacteria are able to live on aerobic or anaerobic conditions.

Urease test is performed to determine the ability of bacteria in decompose urea which produces the enzyme urease. Urea CO $(NH_2)_2$ and ammonium sulfate (NH_4) $2SO_4$ is a source of nitrogen necessary for growth of microorganisms and enzyme secretions. VP test used to determine the ability of a microorganism which capable to produce 2,3-butanediol as a result of fermentation of glucose. A positive result is indicated by the formation of a red color due to the bonds between guanidine and 65 diacetyl (Cappuccino &

Table 1. The macroscopic characteristic of cellulolitic bacteria on Black Soldier larvae gut, with CMC medium

Isolate	Shape	Size	Elevation	Margin	Colour
2.3.A	Circular	Small	Raise	Entire	White
1.4.A	Irregular	Small	Flat	Filamentous	Cream
1.4.C	Irregular	Small	Flat	Entire	Cream
1.5.A	Circular	Large	Flat	Filamentous	Cream
3.6.B	Rhizoid	Medium	Flat	Serrate	Cream
1.8.B	Circular	Large	Flat	Serrate	Cream
1.9.A	Circular	Large	Flat	Undulate	White
1.9.B	Circular	Medium	Flat	Serrate	White
3.10.A	Circular	Small	Flat	Lobate	White

Table 2. Characteristic microscopic and test of cellulolytic biochemical bacteria from larval gut black soldier

Larvae were fed with rice straw								
Isolate	Shape	Gram	Catalase	Endospore	Glucose	Mannitol	Citrate	Motility
2.3.A	Basil	+	+	+	+	+	+	+
1.4.A	Basil	-	-	-	+	-	-	+
1.4.C	Basil	+	+	+	+	+	-	+
1.5.A	Basil	-	+	-	+	+	-	+
3.6.B	Coccus	+	+	-	+	+	+	+
1.8.B	Basil	+	+	+	+	-	-	+
1.9.A	Basil	+	+	+	+	-	-	+
1.9.B	Basil	+	+	+	-	-	+	+
3.10.A	Basil	+	+	+	-	-	-	+

Note: + producing bubbles of gas
not producing bubbles of gas

Table 3. Celuloticindeks of bacteria on *Black Soldier* larvae gut. with CMC medium

Isolate	Diameter of transparent zone (dz) (mm)	Diameter of coloni (dk) (mm)	Cellulolitic activity (dz/dk)
2.3.A	3.8	1.8	2.1
1.4.A	2.7	2.2	1.2
1.4.C	4.1	3.8	1.1
1.5.A	3.1	2.3	1.3
3.6.B	4.7	2.7	1.7
1.8.B	3.4	2.5	1.4
1.9.A	4.7	3.7	1.2
1.9.B	2.7	1.8	1.5
3.10.A	3.8	2.6	1.4

Sherman, 2008). Simon test citrate performed to determine the use of citrate as a carbon source by bacteria (Lay, 1994).

Based on the data in Table 4, it shows that the differences of the cellulolitic index because the cellulose that excreted by each of the different isolates bacterial with the different potential to decompose substrate in the growth medium. The larger the index cellulase in isolates, the greater the result of cellulolytic activity (Apun et al., 2000).

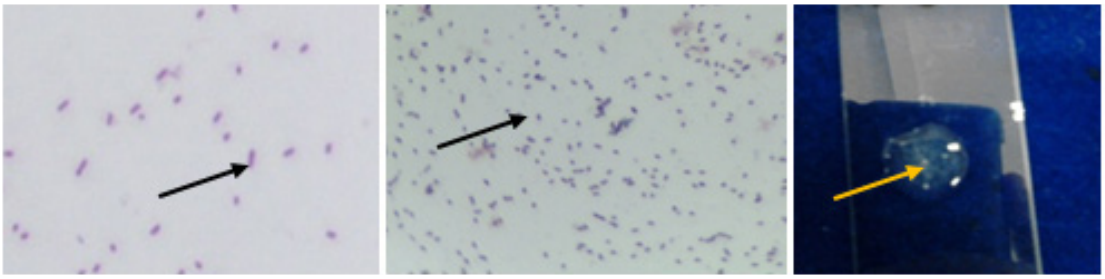
Cellulolytic bacteria's ability to degrade cellulose, for their enzyme cellulase which is able to decompose cellulose in 1,4-glycosidic bond into simpler compounds, namely glucose. Based on the results of biochemical tests, it is assumed that mostly are *Bacillus* sp bacteria namely isolates number 1, 3, 5, 6, 7, 8, and 9.

Table 4. Cellulolytic bacteria in *Black Soldier* larvae gut

Isolate	Species
2.3.A	*Bacillus* sp.
1.4.A	*Proteus* sp.
1.4.C	*Bacilus thruiengensis*
1.5.A	*Proteus* sp.
3.6.B	*Ruminococcus* sp.
1.8.B	*Bacillus thruiengensis*
1.9.A	*Bacillus thruiengensis*
1.9.B	*Bacillus* sp.
3.10.A	*Bacillus* sp.

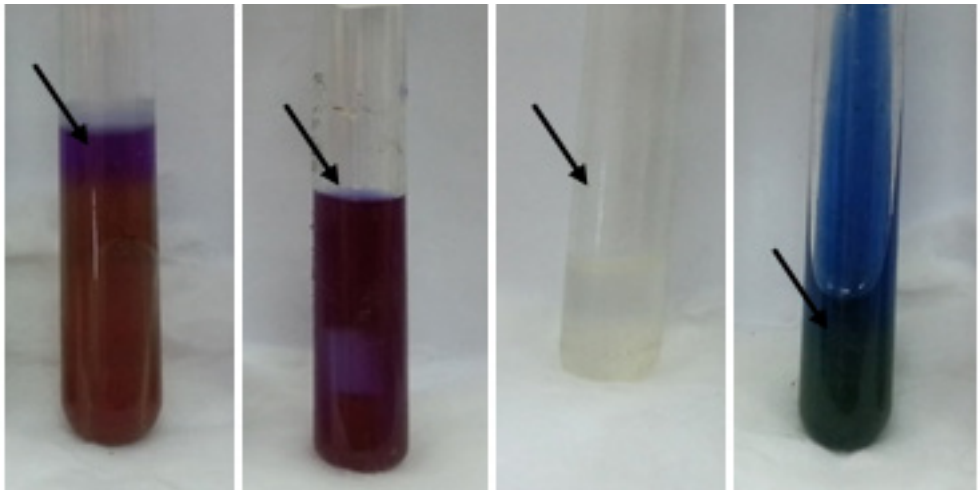
Identification of cellulolytic bacteria

Based on Table 5, it was obtained nine types identified cellulolytic bacteria from the gut of



A B C

Figure 2. Basil shape (A), endospore + (B), catalase + (C) *Bacillus* sp. on CMC medium (1000 x)



A B C D

Figure 3. Glucose test (+) (A), manitoltes (+) (B), semi solid test (+) (C), and simon citrate test (+) (D), of *Bacillus* sp with incubation 37 ^{0}C for 24 h.

the larvae. *Bacillus* sp. is a bacteria that have the highest potential as larvae in the gut cellulolytic Black Soldier.

The pattern of selected cellulolitic bacterial isolates growth

The growth pattern of isolates bacteria CMC medium indicates that the medium is capable to supply nutrients for bacterial cell growth well. The growth of isolate bacteria occurred between the hours-0 and continue to increase the number of cells up to 48 hours (Figure 2)

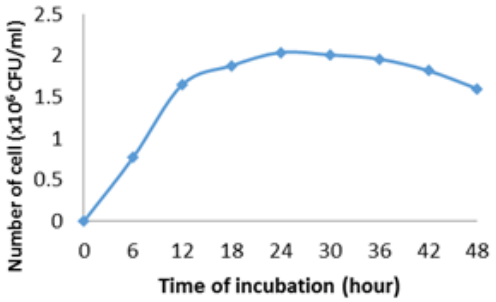


Figure 4. The growth of *Bacillus* sp. *in* CMC medium

Figure 4, shows that the bacteria undergo logarithmic phase from the 6th hour till the 12th with a cell number of 0.77 x 106 CFU/ml and 1.65 x 106 CFU / ml. at the 18th hour until the 36th hour, the bacteria is in stationary phase with the number of bacteria in consecutive namely 1.88 CFU / ml, 2.04 x 106 CFU / ml, 2.01 x 106 CFU / ml, 1.96 x 106 CFU / ml. while on the 42nd hour and 48th hour, the bacteria undergo the death phase by the number of bacterial cells that is 1.82 x 106 CFU / ml and 1.6 x 106 CFU / ml. In the logarithmic phase, the number of cell are rapidly increased until the specific limit so that get into the static phase, maximum cell metabolism and synthesis of cell material quickly with constant until nutrient depleted. At the 18th until the 36th hour, it entered the static or constantphase, it is suspected that the nutrients in the medium began to decrease. The decline of accelerated growth called *decelerated phase*. At the 42nd until the 48th hour, the bacteria through death phase, it is assumed that the nutrients in the medium have been used up for bacterial growth process.

Enzym activities

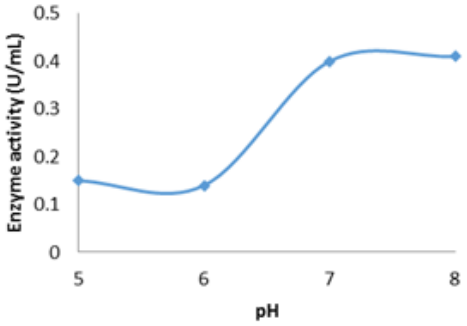


Figure 5. The curve of *Bacillus* sp. cellulose enzym on pH treatment variation

The above graph shows cellulase enzyme activity at an incubation temperature of 40 ^{0}C. Value of enzyme activity based on treatment of pH 5, 6, 7, and 8 respectively are0:15 (± 0.002), 0:14 (± 0.004), 0.4 (± 0.004), and 0:41 (± 0.001) (U / mL). The optimum enzyme activity at pH 7 and 8 temperature is 0.4 (± 0.004), and 0:41 (± 0.001). The enzyme has optimum activity at a certain pH, but mostly optimum enzyme is at neutral pH. Enzymes have active groups that have positiveand negative contain. The enzyme activity will be optimum if there is a balance between the two contains (Pelczar et al., 2000).

In this study, we have found several cellulolytic bacteria that act as agents of degrading cellulose. the presence of these bacteria help the black soldier fly larvae in digestion process of organic material. the ability of larvae to degrade organic waste in the gut because there is a digestive enzyme, cellulose, that is produced by cellulolytic bacteria. The results of this study can provide information that occurs between bacteria symbiosis with larvae of the Black soldier, that bacteria in the gut of insects are able to degrade and metabolize cellulose into acetate, hydrogen and carbon dioxide. bacteria then exploit H_2 and CO_2 to form additional acetic acid. there is also methanogens can use H_2 and CO_2 to form methane and hydrogen transfer between species to remove the excess hydrogen, which can be toxic to organisms at higher concentrations (Ohkuma, 2003)

CONCLUSIONS

There are 9 cellulolitic isolates bacteria based on the observation of macroscopic, microscopic and biochemical tests. The isolates are assumed to be *Bacillus* sp. bacteria (isolates 1, 3, 5, 6, 7, 8, and 9), *Proteus* (isolate number 2 and 5), *Ruminococcus* (isolates number 6). Isolates number 1 is a bacteria *Bacillus* sp. The most potential based on its cellulolytic index is 2.1 mm. The bacteria is optimum at pH 7 and 8, with activities 0.4 and 0.41 U / mL.

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