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Morphological and Biochemical Characteristic of Endosymbiont Cellulolytic Bacteria from Gut of *Hypothenemus hampei* Ferr. and Its Enzyme Activity

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Abstract. *Hypothenemus hampei* Ferr. is one of the most destruction pest in coffee berry. To control this pest by synthetic insecticide is harmful and damage for environment. A strategy to control *H. hampei* is by studying its life cycle. The egg stage until adult of *H. hampei* lives inside coffee beans and utilize polysaccharides of coffee beans as source of its metabolism. The result of investigation showed that in gut of *H. hampei* was found cellulolytic bacteria Isolate 10 (ISH 10) which is suspected as endosymbiont bacteria that helps in the digestive process and metabolism of *H. hampei*. Morphological characters of ISH10 colony are circular, flat elevation, edge of entire colony, and the cell shape is rod. ISH 10 was gram positive bacteria which gave positive results in catalase and negative for oxidase test. In addition, ISH 10 is able to ferment some sugars media test such as glucose, sucrose, fructose and mannitol. ISH10 produced positive reaction in simmon citrate and urease test, and ISH 10 unable to reduce nitrate. Based on results, ISH 10 similar to genus *Brochothrix*. ISH 10 can also produce cellulase with the highest activity of the crude enzyme 0,031 U/ml only in 72 hours of incubation at 30°C against 0,5% CMC in 20 mM acetate buffer pH 5. Further characterization of this cellulase is needed so that it can interfere the digestion of *H. hampei* through endosymbiont cellulolytic bacteria by using inhibitor or environmental factor of its cellulase enzyme.

INTRODUCTION

Coffee is one of important comodities in Indonesia. Coffee production in Indonesia is around 6,6% of production in the world, so that making Indonesia as the third largest coffee producing country [1]. However, in recent years (2013-2018) coffee production in Indonesia has declined significantly. This significant decline caused the decline in Indonesia's position as the third coffee producer in the world to become the fourth position in the world after Colombia [2]. One of the reasons for the decline in coffee production is caused by the activity of coffee berry borer (*Hypothenemus hampei* Ferr.).

H. hampei is a main and most destructive pest in coffee beans. Adult females of *H. Hampei* make hole on coffee fruit and lay eggs in the coffee fruit. After the eggs hatch, the larvae of *H. hampei* eat the endosperm of coffee beans. Feeding activity of this pest causing a decrease in the quality and quantity of coffee fruit up to 40% [3]. All life cycle of *H. hampei* occur inside of coffee beans even though there are some toxic content in coffee such as caffeine

So far, synthetic insecticide is used to control *H. hampei*. The use of synthetic insecticide can cause some problems such as resistance and resurgance of the pest, water and soil pollution, as well as threats to human health [4]. One of the alternative to change synthetic insecticide is the use of botanical insecticide. The use of botanical insecticide is more environmentally friendly, however the results of botanical insecticide application are relatively longer, so that it is still a problems among coffee farmers. Therefore need another way to control this pest that the entire life cycle occurs inside coffee bean with control through its metabolism.

Life cycle of *H. hampei* which is dominant in coffee beans with high caffeine content shows that this insect has high adaptability and tolerance to environmental conditions in coffee beans. This ability is due to symbiotic between *H. hampei* with degrading bacteria in the digestive tract. One of endosymbiont bacteria in *H. hampei* is caffeine

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degrading bacteria such as group of *Pseudomonas* [5]. In addition to endosymbiont bacteria that help to degrade caffeine, *H. hampei* also need a simple carbon source for its metabolism. Simple carbon source can be obtain by breaking down polysaccharide substrate into simple monomer that can be absorbed by *H. hampei*. One of simple carbon source in coffee fruit is cellulose. Cellulose content of robusta coffee is 32-42%, while in arabica coffee is 41-43% [6].

Research on endosymbiont cellulolytic bacteria in gut of *H. hampei* has never been done, so that the aim of this study are to identified endosymbiont cellulolytic bacteria from gut of *H. hampei* based on morphological and biochemical characteristics and to know the activity of its cellulase enzyme. This research will be very useful as a study for the new strategy of handling *H. hampei*.

MATERIALS AND METHODS

H. hampei Collection

H. hampei was obtained from Coffee plantation Durjo, District of Jember. *H. hampei* was pooled from infected coffee berry. Collected *H. hampei*, were killed and disinfected using alcohol 70% to avoid external contamination.

Isolation of Endosymbiont Cellulolytic Bacteria

To dissect *H. hampei*, the methods of Morales-Jimenez *et al*., [7] with slight modification was employed. Ten *H. hampei* specimens were submerged in 100 µl sterilized phosphate buffer solution (PBS) pH 7 50 mM to avoid external contamination. Adults of *H. hampei* were dissected under sterile condition. Gut of *H. hampei* were obtained by removing the elytra, wings and pulling the last segmen of its abdomen. The guts were transfered to 1,5 ml microcentrifuge tube with 200 µl PBS. Gut extraction was crushed with sterile micropistile and suspended using the vortex about 5 minutes. Suspension was diluted in phosphate buffer at a proportion of 1:10. Fifty microlitres of suspension was inoculated on CMC (*Carboxymethyl Cellulose*) agar plate (CMC 0,5%; NaH_2PO_4 6 g; KH_2PO_4 3 g; NaCl 0,5 g; Mg_2SO_4 0.25 g; NH_4Cl 1 g; and agar 23 g), and then incubated at 37^{o}C for 5-7 days [5]. A single colony of bacteria grown on CMC media was recultured on Nutrient Agar (NA) medium to obtained pure isolate by picking up one loop of a single colony. Every pure culture of the bacteria was maintained on slant medium as stock bacteria culture and stored at 4^{o}C for further analysis [8]. Each bacteria isolate were given code ISH (*Isolat Selulolitik Hypothenemus hampei*) with number on each isolates.

Cellulolytic Degrading Activity Assay

The experiment was adapated from Arimurti *et al*. [9]. Each cellulolytic bacteria was inoculated into 10 mL of Nutrient Broth (NB) as many as one loop each isolates, and then incubated overnight. CMC media 0,5 % on plate was made holes by cork borer 0,5 cm. 20 µl culture suspension with equal cell density (1,9 optical density/OD at 600 nm) were filled into each hole then incubated at 30^{o}C for 3 days.

Cellulolytic activity based on clear zone can be known by flooding 10 mL iodin 0,33% on the agar plate for 5 minutes [10]. A clear zone which surrounded the hole were observed and measured using calipers. A clear zone indicated as crboxymethylcellulose degrading activity. Values of Cellulolytic activity index can be calculated using the following equaton [11].

$$\text{Cellulolytic Activity Index} = \frac{\text{colony diameter with clear zone (mm)}}{\text{colony diameter (mm)}} \qquad (1)$$

Bacterial isolates with the highest cellulolytic acttivty (the largest clear zone) were selected to identified and testes the produced enzyme activity .

Identification of Selected Bacteria

Identification of selected cellulolytic bacteria was based on morphological and biochemical characters. Morphological characters include colony shape, elevation, edge of colony, and cell shape. Biochemical characters include gram staining, catalase, oxidase, motility, carbohydrate urease, indol, reduction of nitrate, and simmon

citrate test. Observations results were compared using *Bergey's Manual of Determinative Bacteriology 9th, Microbiology: A Laboratory Manual*.

Cellulase Production

Production of cellulase methods were adapted from Bakare *et al*., [12]. The selected cellulolytic bacteria was cultured in CMC 0,5% Broth and incubated for 24 h on shaker (120 rev/min) at room temperature. Crude enzyme was obtained by centriguging the culture at 8.000 rpm for 10 min [13]. The supernatan is used for testing the specific activity of cellulase enzyme.

Optimization of crude cellulase enzyme production

Optimization of the enzyme was carried out to determine the optimum incubation time in producing cellulase enzyme on CMC media by selected cellulolytic bacteria. Crude enzyme was harvested every 24 hours for 3 days. However after harvesting at 48 hours, the harvesting was done at 60 hours. Crude enzyme obtained were tested for specific activity of cellulase based on their reducing sugars by Nelson-Somogy method.

Specific activity of cellulase were carried out based on Nelson [14] that have been modified. 500 µl of 0,5 % CMC dissolved in 20 mM phosphate buffer (pH 5) was mixed with 100 µl of the crude enzyme, and incubated at 37°C for 2 h. After that 500 µl Somogy reagent were added, then boiled for 15 minutes. The negative control was prepared by adding boiled enzyme. After the solution was cold, added 500 µl Nelson reagent and 2,5 ml H_2O. The solution were centrifuged at 8.000 rpm for 10 min. 1 ml sampel was used for absorbance measurement using UV-VIS spectrophotomether at 500 nm. The absorbance value converted to glucose concentration based on standart curve [15].

Specific activity of cellulase was determined using the reduced sugar levels. This activity is known in U/ml. One unit of cellulase enzyme activity means the amount of enzyme needed to realease 1 µmol reducing sugar (glucose) per minute, said to be unit per milliliter [9]. Cellulase activity was calculated using the following equation

$$\text{Cellulase activity } \left(\frac{U}{ml}\right) = \frac{\text{Reducing sugar values x dilution factor}}{V \text{ x } t \text{ x } BM} \qquad (2)$$

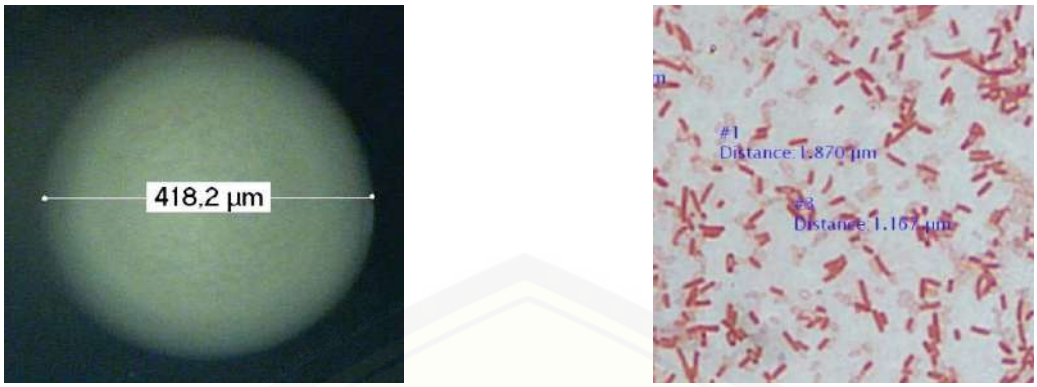
Note : V = volume of enzyme (0,1 ml)
t = time of incubation (120 min)
BM = molucule weight of glucose (180 g/mol)

RESULTS AND DISCUSSION

The results of the bacterial isolation on CMC media were obtained 17 bacterial isolates that were able to grow. The 17 isolates are endosymbiont bacteria in gut of *H. hampei* that is possible to produce cellulase enzymes. All 17 isolates were tested their cellulose degrading activity based on clear zone formed in CMC media. Based on clear zone test, only 5 isolates showed high degrading actvity from 17 isolates. One of selected cellulolytic bacteria that have been characterized is ISH 10.

Morphological and Biochemical Characteristics of ISH 10

Based on macroscopic morphological characters of ISH 10 colony are circular, flat elevation, edge of entire colony. Colony diameter of ISH 19 IS 418,2 µm (Fig 1 a). Cell shape of ISH 10 based on microscopic observation showed that the cell is rod with length 1870 µm (Fig 1 b).



(a) (b)

FIGURE 1. Macroscopic and microscopic morphological characters of ISH 10, (a). Colony characteristic of ISH 10 on nutrient agar media 24 hours; (b). Cell shape of ISH 10, 1000x magnification, Olynmpus BX53 F Microscope

Based on biochemical tests, ISH 10 was gram positive bacteria with not slimy KOH test results shown. ISH 10 showed positive result for catalase test with the appearance of bubbles in this test. This is showed that ISH 10 can produce catalase that can break down H_2O_2 to H_2O and O_2 [16]. Beside that ISH 10 showed negative result of oxidase test. It means that ISH 10 does not produces oxidase enzyme which is able to react with oxygen in the respiration process [16]. 1SH 10 gave positiv reaction to some media in carbohydrate test such as glucose, sucrose, fructose, and mannitol. This is indicated by the change in color of the media from red to yellow. The change means that ISH 10 was able to ferment some sugar media test. Microbial fermentation is a biochemical activity that is carried out by microbes which convert organic macromolecul compounds into simpler compounds in anaerobic conditions. Carbohydrate fermentation can produce various acidic compound such as lactic acid and propionic, ester, ketons, and gas [17].

Result of simmon citrate test showed that ISH 10 can utilize citrate as its carbon source by forming blue in test media. Based on Hemraj *et al*, [16] when the colour of medium changed from green to blue, this indicated that microorganisms can utilize citrate as their carbon source. In urease test, ISH 10 gave positive reaction only 21-79 % strain positive by the change color of the media to yellow from red only 20%. ISH 10 gave negative reation in nitrate and indol test. This showed that ISH 10 unable to reduce NO_3 to NO_2 [18], and ISH 10 can not degrade amino acid tryptophan [17]. Based on identification results, ISH 10 similar to genus *Brochothrix* (Table 1.)

Genus *Brochrotrix* belongs to family Lactobacillaceae. Genus *Brochrotrix* has regular, unbranched rods cell shape with the lenght 0,6-0,7 x 1-2 µm and occurs singlu, in chains, or in long filamentous chains that fold into knotted masses. Coccoid forms appears in old cultures. Genus *Brochrotrix* is gram positive, the cells are not encapsulated, are nonmotile, and are nonsporing. *Brochrotrix* species are facultative anaerobes and are nonpigmented. They grow at a temperature of 0-30ºC (optimum 20-25ºC). The major fermentation product from glucose is $_{L(+)}$-lactate. Cells are catalase positive and contain cytochromes. They stain methyl red and Voges-Proskauer positive. *Brochrotrix* species occur mainly in meat product but are widely distributed in the environment [19].

TABLE 1. Comparison of ISH 10 morphological and biochemical characteristics with certain bacterial isolates

Characteristics		Results	*Brochothrix* (Holt, 1994)	*Carnobacterium* (Holt, 1994)
Gram		-	-	-
Cell shape		Rod	Slender rods, often filaments	Coccus
Motility in liquid media		-	-	D
Oxidase		-	-	-
Catalase		+	+	-
Acid from breakdown of carbohydrate (fermentation product)		+	+ (Mainly lactate)	+(*Mainly* $_{L(+)}$*-lactate*)
Carbohydrate Test	Glucose*	+(A)		
	Sucrose*	+(A)		

Characteristics	Results	*Brochothrix* (Holt, 1994)	*Carnobacterium* (Holt, 1994)
Fructose*	+(A)		
Maltose*	-		
Arabinose*	-		
Lactose*	-		
Mannose*	+(A)		
Spore	NT	-	-
Nitrate reduction	-	-	-
Simmon Citrate Test*	+	-	-
Urease Test*	d	-	-
Indol Test*	-	-	-

Notes : NT : non testes; + : 100-80% strain positive; - : 20-0% strain positive; d : 79-21% strain positive; D : substantial proportion of different species; +(A): positive fermentation with formed acid; * : not as a genus determining character in the identification book

Optimization of crude cellulase enzyme production ISH 10

Optimization of the enzyme was carried out to determine the optimum incubation time in producing cellulase enzyme on CMC media. ISH 10 produced crude enzyme with the highest activity (0,031 U/ml) at 72 hours incubation time (Fig. 2). The crude enzyme activity of ISH 10 is small because the activity value is less than 0,1 U/ml. thi is different from cellulase enzymeproduced by *Bacillus* sp. Based on the research of [20] showed that the result of cellulase crude enzyme activity produced by *Bacillus cereus* were 0,104 U/ml.

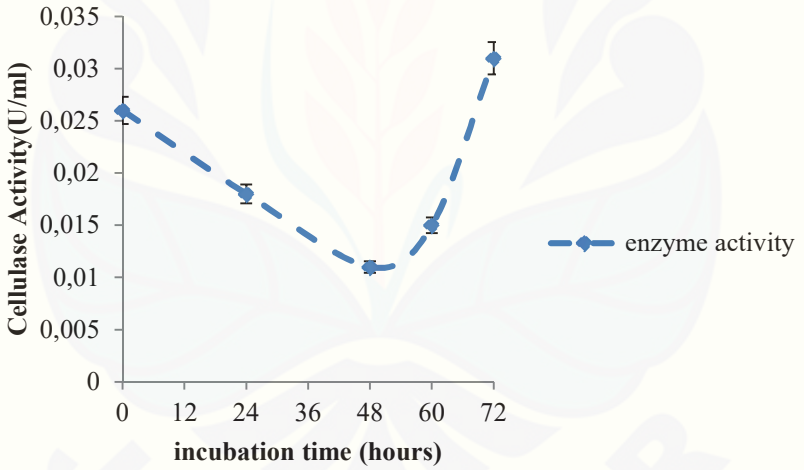


FIGURE 2. Curve of activity enzyme from the optimization of cellulase crude enzyme by ISH 10

This result indicated that ISH 10 has cellulase activity although the value is small. Some species of *Brochotrhix*, *Brochotrhrix thermosphacta* was known to be able to hydrolyze cellulose [21]. This showed that some species of *Brochothrix* are able to produce cellulase, but no research has been conducted to find out specific activity of this enzyme. This resulte can be used as prelimenary research on alternative control of *H. hampei*. Based on Berasategui *et al*., [22] and Macedo and Freire [23], knowledge about interaction between endosymbiont microbe and host insect can be explioted in two different ways. One way is by targeting or utilizing symbiotic interaction to control agricultural pests. This control can be done from microbial interactions or by damaging insect digestive enzymes.

CONCLUSION

ISH 10 is an endosymbiont cellulolytic bacteria from gut of *H. hampei*. Based on the morphological and biochemical characteristics, ISH 10 is similar to genus *Brochothrix*. ISH 10 has highest cellulase activity 0,031 U/ml in 72 hours of incubation time at 30ºC in broth medium of CMC 1%. This results indicate can be used as prelimenary research on alternative control of *H. hampei* Further characterization of this cellulase is needed so that it can interfere the digestion of *H. hampei* through endosymbiont cellulolytic bacteria by using inhibitor or environmental factor of its cellulase enzyme.

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