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Cellulolytic and hemicellulolytic bacteria from the gut of *Oryctes rhinoceros* larvae

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Abstract. *Sari SLA, Pangastuti A, Susilowati A, Purwoko Tj, Mahajoeno E, Hidayat W, Mardhena I, Panuntun DF, Kurniawati D, Anitasari R. 2016. Cellulolytic and hemicellulolytic bacteria from the gut of* Oryctes rhinoceros *larvae. Biodiversitas 17: 78-83.* Lignocellulose is very potential as raw material for biofuel production because it is cheap, abundant and renewable. The main carbohydrate constituents of lignocellulosic material are cellulose and hemicelluloses (a group of heteropolymers that includes xylans and mannans). The most important process in bioethanol production from lignocellulose is the bioconversion of polysaccharides into fermentable sugar. Enzymatic hydrolysis has been developed because it is the more environmentally approach. Since the cost of hydrolytic enzyme production is the major problem of the process, many type of research has been focused on lowering the cost of enzyme production, including screening for organisms with a novel enzyme. This present study was conducted to isolate and screen of the cellulolytic and hemicellulolytic Bacteria from the gut of *Oryctes rhinoceros* L. larvae. The 3rd instars were used in this research. The research succeeded to isolate 11 bacterial isolates from the gut of *O. rhinoceros* larvae. The screening result demonstrated that bacterial isolates had cellulolytic (63.6% of total isolates), xylanolytic (72.7% of total isolates), and mannanolytic (100% of total isolates) activity. Based on the 16S rDNA sequence, 10 isolates were classified into *Bacillus* and only 1 isolate was classified into *Citrobacter*. The GOR2 which was closely related to *Bacillus pumilus* vit bac1 has the highest cellulolytic and xylanolytic activities. The isolate with the highest mannanolytic activity was the GOR7 which was closely related to *Bacillus aryabhattai* strain IHB B 6821.

Keywords: Cellulolytic, hemicellulolytic, lignocellulosic, *Oryctes rhinoceros*

INTRODUCTION

Increasing worldwide demand for energy, fuel oil depletion, continuously price increasing of crude oil and environment issues such as global warming and pollution encourage intensive investigation to develop an alternative renewable energy **(**Dashtban et al. 2009). Bioethanol is an alternative energy source that can be potentially developed to replace petroleum. It can be utilized as transportation fuels with little change to current technology, renewable and reduce green house gas emissions (Carere et al. 2008).

Bioethanol can be produced from the organic material which contains sugar, starch, or lignocellulose. Lignocellulose is the main component of plant cell wall. It is very potential to be developed as a substrate for bioethanol production since it is very cheap, abundant, and renewable. Structurally, it is formed of three main polymers, namely: cellulose (a homopolymer of D-glucosyl residues), hemicelluloses (a group of heteropolymers that includes xylans and mannans), and lignin (a complex polyphenolic polymer). The main polysaccharides of lignocelluloses, celluloses and hemicelluloses (mannan, and xylan), can be hydrolyzed to yield fermentable sugars, which can then be fermented to generate bioethanol (Gong et al. 1999; Cheng and Timilsina 2011).

Polysaccharides hydrolysis can be performed either thermochemically using acid and heat or biologically using hydrolytic enzymes. Enzymatic hydrolysis possesses several advantages such as its efficiency is quite high, its byproduct can be controlled, its process does not need expensive instruments, and its energy need is quite low (Badger 2002). However, the enzymatic digestion of native plant cell walls is inefficient, presenting a considerable barrier to cost-effective biofuel production (Lacayo et al. 2013). Much research efforts have been focused on lowering the cost of enzyme production. Various ways are done start from microbial exploration as a potential enzyme source, strain and enzyme engineering, process engineering, including substrate selection, cultivation condition and bioreactor design (Badger 2002; Howard et al. 2003).

Cellulase and hemicellulase are hydrolytic enzymes which play an important role in lignocellulose hydrolysis. Cellulase is a group of enzymes that degrade cellulose, a major component of lignocellulose. Components of cellulase systems were classified based on their mode of catalytic action into three major types: endoglucanase (EC 3.2.1.4), exoglucanase or Cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) (Lynd et al. 2002; Dashtban et al. 2009). Mannanase and xylanase are the key enzymes which have roles in hemicellulose hydrolysis.

Polysaccharide hydrolysis is the key process of herbivorous insect feed digestion (Shi et al. 2011). The herbivorous insect can utilize lignocellulose as its energy source. Its ability is supported by the presence of microbes in its gut, which produce hydrolytic enzymes (Suh et al. 2003). *Rhinoceros* beetles (*Oryctes rhinoceros* L.) is a herbivorous insect belong to the Coleopteran order (Scarabaeidae family) (Bedford 1974; Lavelle et al. 1997; Swamy and Deesh 2011). The *O. rhinoceros* larvae growth in a pile of decaying vegetation and using the residue from organic material as feed (Schmaedick 2005). This research was conducted to isolate and screen cellulolytic, hemicellulolytic (xylanolytic and mannanolytic) bacteria from the gut of *O. rhinoceros* larvae.

MATERIALS AND METHODS

Organism

Oryctes rhinoceros larvae were collected from rice stalk composting field in Srumbung, Magelang, Central Java, Indonesia. The 3rd instars were used in this research**.**

Media

Media for isolating bacteria from *O. rhinoceros* gut was Lauria-Bertani Agar (LA) with composition: 1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 1% agar. Regeneration of bacteria used Lauria-Bertani medium. Screening of cellulolityc bacteria used minimal media (Berg's et al. 1972) with the addition of 10 g L^{-1} carboxymethyl cellulose (CMC) sodium salt low viscosity (Sigma-Aldrich) for cellulolytic, Locus bean gum for mannolytic and 1% beech wood xylan (Sigma-Aldrich) for xylanolytic and pH was adjusted to 7. Composition of minimal medium (in g/100 mL) was 0.2 g $NaNO_3$, 0.05 g $MgSO_4$, 0.005 g K_2HPO_4, 1 mg $FeSO_4$, 2 mg $CaCl_2$, 0.2 mg $MnSO_4$, and 2% agar.

Isolation of bacteria from the gut of *O. rhinoceros* larvae

The 3rd instars were used in this research. The instars were cleaned externally with 95% ethanol and dissected. The entire digestive tract was aseptically isolated in a UV laminar flow hood and homogenized for 5 minutes in sterile NaCl (0.85%) solution then incubated for 30 minutes at 37ºC. Isolation of bacteria was done by dilution plate method. The supernatant was serially diluted 10^3-10^6. After serial dilution, 0.1 mL of solution was taken using sterile micropipette and plated on LA medium. Incubation was done at 37ºC for 48 hours.

Screening for cellulolytic, xylanolytic and mannolytic bacteria

Bacterial isolates were grown in LB medium with the addition of 1% carbon source at 37ºC for 24 hours. Screening for hydrolytic activity was done by inoculating 0.5 µl inoculums into screening medium and incubated at 37°C for 48 hours. The clear zone around colony showed cellulolytic, xylanolytic or mannolytic activity. The clear zone was made become clearer by coloring with Congo red for cellulolytic and xylanolytic (Wood 1980) and Iodin for mannanolytic.

Identification of bacteria

Bacterial isolates were grown in LB medium at 37°C for 24 h. Cultures were centrifuged at 10,000× g for 1 min, and the supernatant was removed. DNA extraction was performed using a Presto™ Mini gDNA Bacteria Kit (Genaid, Taiwan) according to the manufacturer's instructions. Bacterial universal primers 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1387R (5'-CCCGGGAACGTATTCACCGC-3') were used to amplify the 16S rDNA from genomic DNA (Marchesi et al. 1998). Polymerase chain reaction (PCR) was performed in a Thermo Cycler (Aplied Biosystem). The amplification was performed as follows: initial denaturation for 5 min at 94°C, 35 cycles each of denaturation for 15s at 94°C, annealing for 15s at 55°C, and elongation for 15s at 72°C, and a final extension for 7 min at 72°C. PCR product purification and sequencing were done by 1st BASE (Singapore). Sequences of 16S rDN were compared to the 16S rDNA sequences available in the Gen Bank data base using the BLAST program at National Center for Biotechnology Information http: //www.ncbi.nlm.nih.gov/

Phylogeneticanalysis

Phylogenetic analysis was performed using the neighbor-joining method with MEGA 6.0. (Tamura et al. 2013). To statistically evaluate the branching, bootstrap analysis was carried out with data resampled 1000 times

RESULTSAND DISCUSSION

Isolation and screening of cellulolytic bacteria

The research succeeded to isolate 11 bacterial isolates from the gut of *O. rhinoceros* larvae. The screening result based on the formation of a clear zone around the colony demonstrated that bacterial isolates have cellulolytic (63.6%), xylanolytic (72.7%), and mannanolytic (100%) activity (Table 1). Based on the clear zone diameter, it was observed that the isolates have varied ability in hydrolyzing the carbon source in the forms of CMC, Xylan, and mannan. The clear zones diameter ranges between 0.97-3.03 cm on CMC media, 0.17-4 cm on xylan media, and

Table 1. Screening result of cellulolytic and hemicellulolytic bacteria from the gut of *O. rhinoceros* larvae

Code of isolates	Clear zone diameter (cm)		
	Xylan medium	Mannan medium	CMC medium
GOR1	1.17	1.91	3.01
GOR2	**4.00**	1.32	**3.03**
GOR3	0.17	2.61	1.87
GOR4	1.67	2.51	-
GOR5	1.60	2.53	-
GOR6	-	0.55	2.73
GOR7	2.50	**3.48**	2.90
GOR8	2.67	2.36	1.77
GOR9	2.33	1.04	0.97
GOR10	-	2.83	-
GOR11	-	3.39	-

Table 2. Identity of the cellulolytic and hemicellulolytic bacteria isolated from the gut of *O. rhinoceros* larvae based on 16S rDNA sequences

Code of isolates	Accession	E Value	Identity	Strain of closest match
GOR 1	KF135465.1	0.0	99%	*Bacillus subtilis* strain JK1316S
GOR 2	KC845305.1	0.0	99%	*Bacillus pumilus* strain Vit Bac1
GOR 3	KJ534434.1	0.0	99%	*Bacillus cereus* strain LD147
GOR 4	KJ534462.1	0.0	99%	*Bacillus megaterium* strain RD30
GOR 5	JF512478.1	0.0	99%	*Bacillus thuringiensis* strain Pak2310
GOR 6	KF933659.1	0.0	99%	*Bacillus aquimaris* strain HNS62
GOR 7	KF668459.1	0.0	99%	*Bacillus aryabhattai* strain IHB B 6821
GOR 8	KJ572277.1	0.0	99%	*Bacillus cereus* strain L-3
GOR 9	CP000822.1	0.0	99%	*Citrobacter koseri* ATCC BAA-895
GOR 10	FJ189762.1	0.0	99%	*Bacillus clausii* strain MSB08
GOR 11	JF512478.1	0.0	99%	*Bacillus thuringiensis*strain Pak2310

0.55-3.48 cm on mannan media. The highest cellulolytic and xylalolytic activity was demonstrated by GOR2 while the highest mannanolytic activity was displayed by GOR7.

Identification of bacteria

The gram staining results reveal that almost all bacterial isolates were rod-shaped gram-positive, except for GOR9, which was short rod-shaped gram-negative. All isolates were then identified based on the16S rDNA sequences. The 16S rDNA amplicons of some isolates using bacterial universal primers 63F and 1387R showed in Figure 1. Based on the BLAST analysis (Table 2.), all bacteria had a partial sequence similar to the database from the Gene Bank, having the level of similarity of over 99%.

Phylogeneticanalysis

Phylogenetic analyses placed the 11 isolates in the 2 groups: *Bacillus* and *Citrobacter* (Figure 2). *Bacillus* was the dominant group, including 10 isolates. Branching off this unknown group from *Bacteroides cellulolyticus* strain CRE2 was supported by a 100% bootstrap value.

Discussion

The ability to degrade cellulose and hemicellulose can be measured based on clear zone diameter (Figure 3). The isolate ability on making a clear zone showed that this isolate can hydrolyze polysaccharide as a carbon source or produced hydrolytic enzymes which were secreted to the growth medium. These enzymes degrade β-1,4-glycosidic bond in CMC, xylan and mannan. Nevertheless, clear zone diameters were qualitatively reproducible with the plate overlay technique. Theclear zone is made become clearer by coloring with Congo red and Iodin which strongly interacted with β-1,4-glikosidik bound (Wood 1980; Teather and Wood 1982).

Based on clear zone diameter, it was determined that the potential isolates for cellulase and xylanase producer were GOR2 that was closely related to *Bacillus pumilus* vit bac1. *Bacillus pulmilus* have been reported as cellulase (Kotchoni et al. 2006) and xylanase (Lamid 2006) producer. Several *Bacillus* have been reported to have cellulolytic activity such as *B. Brevis* (Singh and Kumar 1998), *B. pumilus*, *B. amyoliquefaciens* DL-3 (Lee et al. 2008), and *B. subtilis* YJ1 (Yin et al. 2010); and xylanolytic activity, such as *B. subtilis* (Heck et al. 2002), *B. altitudinis* (Adhyaru et al. 2014), and *B. cereus* (Mandal et al. 2012). *Bacillus* is the most dominant bacterium used in enzymes industry because of its ability to produce and secrete an amount of extracellular enzymes (Rastogi et al. 2010).

The bacterial isolate with the highest mannanolytic activity was GOR7, that was closely related to *Bacillus aryabhattai*. *Bacillus aryabhattai* have been isolated by Ray et al. (2012) from the rhizosphere region of *Lemna* sp. from the East Kolkata wetlands. *Bacillus aryabhattai* have the properties of withstanding Cr^{3+} exposure, salinity, stress, and also withstanding high UV exposure. *Bacillus aryabhattai* was also discovered as producer of L-asparaginase (Singh and Srivastava 2014) and protease (Sharma et al. 2014).

The result of the research showed that all isolates had cellulolytic or hemicellulolytic activities. Cellulolytic and hemicellulolytic bacteria are found in most herbivorous insects' gut such as termite (Kuhnigk and Konig 1997),

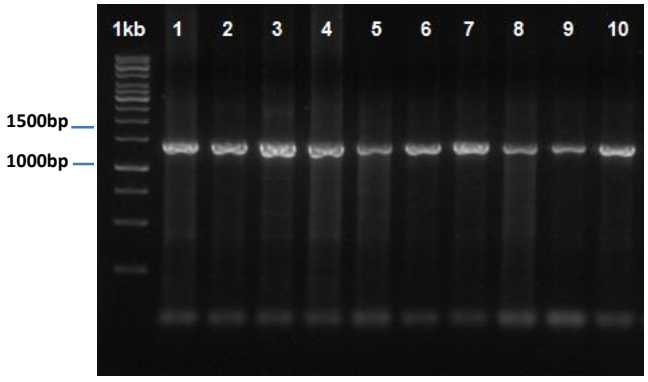

Figure 1. Bacterial 16S rDNA PCR products using Bacterial Universal Primer 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1387R (5'-CCCGGGAACGTATTCACCGC-3'). The following DNA templates were used for PCR (by lane): 1, GOR1; 2, GOR2; 3, GOR3; 4, GOR4; 5, GOR5; 6, GOR6; 7, GOR7; 8, GOR8; 9, GOR9; 10, GOR10

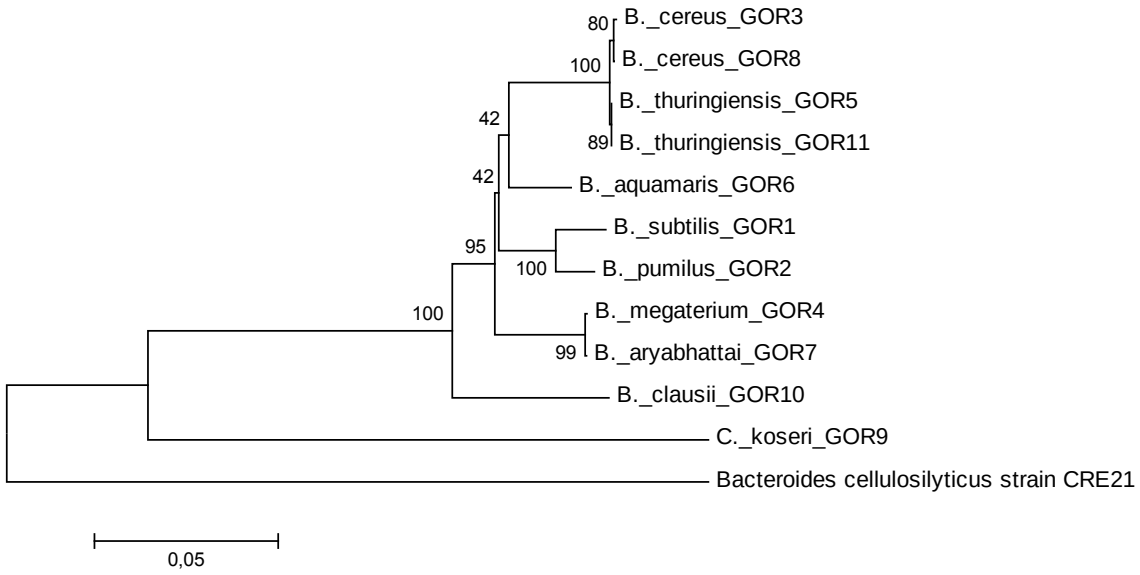

Figure 2.16S rDNA-based dendrogram showing phylogenetic relationships of cellulolytic and hemicellulolytic bacteria from the gut of *O. rhinoceros* larvae (shown with GOR as a code of isolate) to members of the cellulolytic bacteria from Gen Bank data base. Bootstrap values (n=1000 replicates) of ≥49% are reported as percentages. The scale bar represents the number of changes per nucleotide position

Figure 3. Isolate GOR2 on CMC medium (A) and *beech wood xylan* medium (B) after coloring with 1% Congo red and GOR7 on locust bean gum medium after coloring by 0.5 N Iodin (C). The clear zone surrounding a colony indicates hydrolitic activity

Bombix mori larvae (Anand et al. 2010), and *Holotrichia parallela* larvae (Huang et al. 2012). Insects such as *Reticulitermes flavipes* (Zhou et al. 2008), *Anoplophora glabripennis* (Geibet al. 2010), *Tenebrio molitor* (Ferreira et al. 2001), and *Pachnoda marginata* (Cazemier et al. 2003) can degrade plant biomass with the help of microorganism in their gut. The presence of bacteria in the gut of herbivorous insect has an important role in feed hydrolysis. The result of the research demonstrates that the gut of *O. rhinoceros* larvae is an attractive source for the study of novel cellulolytic, xylanolytic and mannanolytic microorganisms and enzymes that are useful for lignocellulose degradation.

The result of the identification shows that 10 out of 11 isolates were classified into *Bacillus*. *Bacillus* is also discovered in the digestive tract of *Holotrichia parallela* (Coleoptera: Scarabaeidae) during the second and third instars (Huang and Zhang 2013) and *Pachnoda* spp. (Coleoptera: Scarabaeidae) (Andert et al. 2010). *Bacillus* can survive in quite high ranges of temperature and pH such as in the gut of Lepidoptera larvae. Lepidoptera larvae have the average temperature around 37^0 C and the alkaline pH so that it is optimum for the growth of *Bacillus* (Broderick, 2003).The bacterial composition in insect's digestive tract also related to the types of feeds (Broderick, 2004).

In conclusion, the research succeeded to isolate 11 bacterial isolates from the gut of *O. rhinoceros* larvae. The screening result demonstrated that bacterial isolates have cellulolytic (63.6% of total isolates), xylanolytic (72.7% of total isolates), and mannanolytic (100% of total isolates) activity. Based on the 16S rDNA sequence, 10 isolates were classified into the *Bacillus* and only 1 isolate was classified into *Citrobacter*. The GOR2 which was closely related to *Bacillus pumilus* vit bac1 has the highest cellulolytic and xylanolytic activities. The isolate with the highest mannanolytic activity was the GOR7 was closely related to *Bacillus aryabhattai* strain IHB B 6821.

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