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Isolation and characterization of cockroach endosymbiont bacteria with potential to produce hydrolytic enzyme of organic material

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ABSTRACT

Cockroaches are one of the most popular decomposers in organic matter processing. Its ability to decompose organic matter is supported by the association with the digestive tract microbiota. Microbial exploration of the cockroach gastrointestinal tract is still rarely studied, especially in Indonesia. This study aims to obtain potential bacterial isolates that produce enzymes that can decompose of organic matter derived from cockroach midgut. Cockroach samples were obtained from household organic wastes composts. Midguts of cockroach sample were surgically removed, mashed aseptically, and inoculated into nutrient agar medium. Endosymbiont bacteria grown in the media then purified, characterized, and tested for enzymatic abilities such as amylase, protease, lipase and cellulase. The abundance of cockroach midgut endosymbiont bacteria was detected as much as 1.1 x 10^4 CFU/mL. A total of 20 endosymbiont bacterial isolates had different characters and had the ability to hydrolyze starch, cellulose, protein, and lipid. Three potential bacterial isolates in hydrolyzing starch, cellulose, protein, and lipid were EKA 4, EKA 8, and EKA 20. Cockroach endosymbiont isolates are potential to be applied for organic waste treatment.

Key words: *Cockroach, Endosymbiont, Enzyme, Midgut, Organic waste*

Introduction

Increasing human population, urbanization, economic growth, biomass production and consumption patterns enhance the amount of solid wastes (Shaoli and Debnath, 2019). Treatment technologies for solid waste can be done by direct use, physicochemical, thermochemical, and biological treatment (Lohri *et al.,* 2017). Biological treatment applied to solid organic waste on a household scale is commonly done by composting (Warunasinghe and Yapa, 2016; Bakari *et al.,* 2017). Composting is the process of decaying organic matter aerobically to produce humus (Epstein, 1996; Sikora, 1998; Lohri *et al.,* 2017). Decomposition is a process of physical or chemical changes in organic matter of animals and plants into simple organic compounds (Susanti and Halwany, 2017). Lohri *et al.,* (2016) revealed that, composting in a household scale is generally done by stacking organic waste in barrels and relying on

the passive aeration process. The main actors in composting organic material are microorganisms such as fungi, yeasts, bacteria and macro invertebrates such as insects (Morales and Wolff, 2010; Lohri *et al.*, 2017). Decomposed organic matter becomes a natural habitat for these organisms (Tuomela *et al.,* 2000). The association of insects in the process of composting organic waste is able to increase the speed of physical and chemical changes of organic waste during decomposition as well as causing a decrease in the molecular weights of compounds (Morales and Wolff, 2010). This makes insects as reliable organisms that can recycle organic matter (Morales and Wolff, 2010; Tarli *et al.*, 2014). Among all insects, cockroaches, member of order Blattodea, are important detritivore in the ecosystem (Evangelista *et al.,* 2013; Mullins, 2015). As detritivores, cockroaches not only consume fresh food types, but even rotten human leftover, and excrement from other cockroaches (Rozendaal, 1997; Moges, *et al.* 2016). The wide variety of organic materials in biological waste products makes the Blattodea order very important for ecosystem functioning (Weiiser and Sieman, 2008; Ifeanyi and Odunayo, 2015).

Cockroaches share their habitat with various types of bacteria which makes association in the form of symbiotic relation is not uncommon. Symbiotic bacteria are transmitted vertically between generations and horizontally from the environment (Bright and Bulgheresi, 2010). Horizontal transmission mainly sourced from organic materials in the habitat (Zhang and Zhang, 2018). Bacteria from the habitat enter the digestive tract of cockroaches and stay for a short time or transient (allochthonous bacteria) and others are able to attach and form colonization of the digestive tract mucus epithelium (autochthonous bacteria). The endosymbiont bacteria showed beneficial effects for the host. In the case of *Blattella germanica*, among others, the endosymbionts increased the host resistant to insecticides (Pietri, *et al.,* 2018), pathogenic fungi (Zhang, 2018), helped nitrogen fixation (Patino-Navarrete, 2014), and helped degrade the complex organic components by producing extracellular enzymes (Tinker and Ottesen, 2016).

Endosymbiont bacteria of cockroach digestive tract work together to alter food (Zhang and Zhang, 2018). Each bacterium can produce different hydrolytic enzymes such as cellulase, amylase, lipase, and protease (Singh *et al.,* 2016). Their enzyme products have the potential to be utilized for various fields in industry. Thus, in this research, we isolated cockroach's endosymbiont and tested their potencies to produce hydrolytic enzymes which can be utilized in various industries.

Endosymbiont diversity in the digestion track of cockroaches is greatly influenced by their diet (Schauer *et al.,* 2012). Omnivorous cockroaches *Periplaneta americana* has a higher endosymbiont diversity compared to wood-feeding cockroach *Cryptocercus punctulatus* (Colman *et al.,* 2012; Pérez-Cobas *et al.,* 2015). Some exploratory research of endosymbiont bacteria in the digestive tract of cockroaches has been carried out including on *Blattella germanica* (Pérez-Cobas, *et al.,* 2015), *Shelfordella lateralis* (Schauer, 2012), *Periplaneta americana* (Tinker and Ottesen, 2016), *Panchlora* (Gontang *et al.,* 2017), and litter-feeding cockroach *Pycnoselus surinamensis* (Richards *et al.,* 2017). However, there is still rare information about the hydrolytic enzymes produced by the endosymbiont bacteria of *Pycnoselus surinamensis*.

In this study, endosymbiont bacteria were isolated from the gut of *Pynocelus surinamensis*. The diversity of bacteria was detected by culture-dependent methods. This study will add the data about diversity of endosymbiont bacteria from gut of *Pynocelus surinamensis* that have ability to produce hydrolytic enzymes of starch, cellulose, protein, and lipid.

Materials and Methods

Cockroach collection

Cockroaches were obtained from Surabaya urban household waste treatment plants which had been acclimated for 2 weeks at the Faculty of Science and Technology, Universitas Airlangga, Surabaya, Indonesia. The most abundant cockroach that was chosen as the object of this study was *Pycnoscelus surinamensis* according to Roth, 1998.

Dissection and endosymbiontbacteria isolation of cockroach

A Cockroach obtained by hand sorting was anesthetized with chloroform, and then the legs were removed. The side of the cockroach was slashed from the posterior to anterior to obtain the intact digestive tract. The digestive tract organ was separated from the body. The digestive tract organ was steril-

ized according to Wynants *et al.* (2018) with modification: digestive tract organ was washed with 10 mL of 70% alcohol then followed by two washes of 10 mL of sterile distilled water. Each washing step was carried out for 15 seconds on a vortex with a speed of 2000 rpm. The gut was cut aseptically, crushed, and dissolved in a physiological saline solution (NaCl 0.85%). One mL of the suspension was diluted at stratified dilution until it reaches a certain bacterial density. Bacteria were grown onNutrient Agar (NA) (Oxoid), Strach Casein Agar (SCA), and de Man, Rogosa, and Shape Agar (MRSA) with pour plate technique and incubated for 24-48 hours at temperatures of approximately 29 ºC.

Quantification and characterization of cockroach endosymbiont bacteria

Bacteria that grow in each medium were counted using the Total Plate Count (TPC) principle. Colonies that have different characters were purified and rejuvenated for hydrolytic assay. Macroscopic characterizations were done by observing the character of bacterial colonies consisting of shape, size, color, margin, elevation, and transparency, while microscopic characterizations were done by Gram staining and observing at shape of bacterial cells.

Hydrolytic assay

Amylolytic, proteolytic, cellulolytic, and lipolytic activities of each isolates were assessed on solid media that had been modified with the test substrates. Hydrolytic activity by amylase-producing potential bacteria was carried out on NA which was added by starch 2% (w/v) (Banerjee and Ghosh, 2014), hydrolytic activity by protease-producing potential bacteria was carried out on NA which was added by skim milk 2% (v/v) (Bhowmik, *et al.*, 2015), hydrolytic activity by cellulase-producing potential bacteria was carried out on Carboxy Methyl Celullose (CMC), hydrolytic activity by potential lipase-producing bacteria was carried out on NA which was added by glycerol 2% (v/v). Incubation was carried out for 24 hours at a temperature of approximately 29 ºC. Visualization of hydrolytic activity of each substrate was different. To determine the presence of amylase was done by adding a 2% iodine indicator solution to the test media, to determine the presence of proteases without the addition of any reagents, to determine the presence of cellulase was done by adding Congo red indicator solution then washed with NaCl 1% solution for 20 minutes, to determine the presence of lipase by adding rhodamine indicator solution which was then observed under UV light. Positive test results were indicated by the presence of a clear zone around the bacterial colony. The hydrolytic activity index was calculated using the formula (Febriyanto *et al.*, 2015):

$$\text{Hydrolytic Activity Index} = \frac{\text{Bacteria Colony Zones Diameter (mm)}}{\text{Bacteria Colony Diameter (mm)}}$$

Identification of potential bacteria based on physiological character

The three enzymatic potential bacteria were identified using Microbact kit GNB 12A and 12 B (Oxoid) based on manufacturer's instructions. Identification of bacteria isolates obtained based on Bergey's Manual of Determinative Bacteriology, Holt (1994).

Results

Quantification and characterization of cockroach endosymbiont bacteria

The abundance of endosymbiont bacteria in the cockroach gut obtained was 1.1 x 10^4 CFU/mL. A total of 20 bacterial isolates have different characteristics of macroscopic and microscopic. One of twenty isolate was difficult to rejuvenate (EKA 14) (data not shown). Macroscopic and microscopic characters of bacteria are shown in Table 1.

Hydrolytic activity

From a total of 20 bacterial isolates, 16 bacterial isolates had amylolytic activity, 15 bacterial isolates had proteolytic activity, 16 bacterial isolates had cellulolytic activity, and 11 bacterial isolates had lipolytic activity. The data is shown at Table 2. Observation of amylase, protease, and cellulase enzymes were carried out quantitatively, while observation the activity of lipase enzymes was carried out qualitatively by observing the presence of zones and fluorescence visible when exposed to UV light.

The results of the hydrolytic activity index showed the average index value of potential isolates was more then 1, with an average standard deviation of ± 10% (Table 2). It shows that the bacteria potential to produce amylase, protease, and celullase. In the hydrolytic assay of amylase, 80% of bacterial isolates showed amylolytic activity with the highest index of 3.84 (EKA 20) and the lowest of 1.03 (EKA 9). As many as 75% of bacterial isolates

Table 1. Macroscopic and microscopic characteristic of cockroach endosymbiont bacteria

Bacteria Codes	Macroscopic Character					Microscopic Character		
	Color of Colony	Shape	Margin	Elevation	Structure	Shape of Cell	Existence of Spore	Gram Staining
EKA 1	Cream	Irregular	Undulate	Raised	Opaque	Rod	+	+
EKA 2	Yellow	Irregular	Undulate	Crateriform	Opaque	Shape Variable	-	-
EKA 3	Cream	Irregular	Entire	Flat	Translucent	Rod	+	+
EKA 4	Cream	Circular	Entire	Crateriform	Opaque	Rod	+	+
EKA 5	Cream	Irregular	Filiform	Raised	Opaque	Rod	+	+
EKA 6	Cream	Irregular	Undulate	Umbonate	Translucent	Rod	+	+
EKA 7	Cream	Irregular	Filiform	Flat	Translucent	Rod	+	+
EKA 8	Cream	Irregular	Undulate	Flat	Translucent	Rod	+	+
EKA 9	Cream	Irregular	Undulate	Flat	Translucent	Rod	+	+
EKA 10	Cream	Irregular	Entire	Convex	Opaque	Rod	+	+
EKA 11	Cream	Irregular	Curled	Flat	Opaque	Rod	+	+
EKA 12	Cream	Circullar	Entire	Convex	Opaque	Rod	+	+
EKA 13	Cream	Irregular	Curled	Flat	Opaque	Rod	+	+
EKA 15	Orange	Irregular	Undulate	Raised	Opaque	Rod	+	+
EKA 16	Orange	Irregular	Undulate	Flat	Opaque	Rod	+	+
EKA 17	Cream	Irregular	Undulate	Raised	Opaque	Rod	+	+
EKA 18	Cream	Irregular	Undulate	Raised	Translucent	Rod	+	+
EKA 19	Cream	Irregular	Lobate	Flat	Translucent	Rod	+	+
EKA 20	Cream	Irregular	Curled	Flat	Opaque	Rod	+	+

Table 2. Hydrolytic activity index of endosymbiont bacterial isolates

Isolate code	Amylase Index	Protease Index	Celullase Index	Lipase Activity
EKA 1	2.39 ± 0.21	2.16 ± 0.17	1.67 ± 0.00	+++
EKA 2	2.66 ± 0.05	-	2.53 ± 0.05	-
EKA 3	1.74 ± 0.07	2.53 ± 0.29	1.58 ± 0.12	++
EKA 4	2.91 ± 0.04	5.94 ± 0.02	2.42 ± 0.34	+++
EKA 5	1.31 ± 0.04	2.16 ± 0.20	1.32 ± 0.13	-
EKA 6	2.10 ± 0.05	1.41 ± 0.02	1.44 ± 0.04	++
EKA 7	2.18 ± 0.12	1.97 ± 0.00	1.84 ± 0.20	+++
EKA 8	2.48 ± 0.14	2.95 ± 0.16	2.63 ± 0.13	+++
EKA 9	1.03 ± 0.05	-	1.47 ± 0.13	-
EKA 10	-	-	-	++
EKA 11	1.88 ± 0.04	-	2.17 ± 0.05	-
EKA 12	-	2.33 ± 0.19	-	+++
EKA 13	1.21 ± 0.03	1.09 ± 0.13	1.23 ± 0.10	-
EKA 15	1.15 ± 0.05	3.21 ± 0.35	1.01 ± 0.07	-
EKA 16	2.02 ± 0.17	4.02 ± 0.09	1.16 ± 0.13	-
EKA 17	1.05 ± 0.04	3.61 ± 0.00	1.11 ± 0.09	+
EKA 18	-	3.18 ± 0.29	-	+
EKA 19	3.30 ± 0.15	2.31 ± 0.08	3.99 ± 0.46	-
EKA 20	3.84 ± 0.01	2.64 ± 0.25	2.43 ± 0.05	+

(-) not detected, (+) fluorescent colony, (++) fluorescent zone, (+++) fluorescent colony and zone

showed proteolytic activity with the highest index of 5.94 (EKA 4) and the lowest of 1.09 (EKA 13). In the hydrolytic assay of cellullose, 80% of bacterial isolates showed cellulolytic activity with the highest index of 3.99 (EKA 19) and the lowest 1.01 (EKA 15). Whereas 55% of bacterial isolates showed lipolytic activity, with potential isolates of 5 isolates (EKA 1, EKA 4, EKA 7, EKA 8, and EKA 12).

Several bacterial isolates were found to have the ability to multi-enzyme. Isolates capable of produc-

ing amylase, protease, cellulase, and lipase enzymes together were 8 isolates; they were EKA 1, EKA 3, EKA 4, EKA 6, EKA 7, EKA 8, EKA 17 and EKA 20. Bacterial isolates that were able to produce amylase, protease, and cellulase are 5 isolates, namely EKA 5, EKA 13, EKA 15, EKA 16, and EKA 19. Isolates that could produce amylase and cellulase are 3 isolates namely EKA 2, EKA 3, and EKA 11. Isolates that were able to produce protease and lipase are 2 isolates namely EKA 12, and EKA 18. Bacteria that have the most potential enzyme multi-activity were EKA 4, EKA 8 and EKA 20. Enzyme multi-activity of cockroach gut endosymbiont bacterial is shown in Fig. 1.

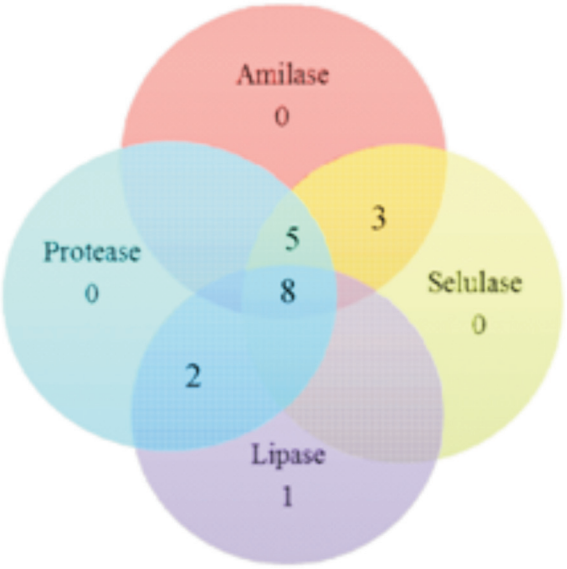

Fig 1. Summary of cocroach endosymbiont bacteria enzimaticpotency

Identification of potential bacteria based on physiological character

Physiological characters of EKA 4, EKA 8, and EKA 20 are shown in Table 3. Based on the book of Bergey's Manual of Determinative Bacteriology, EKA 4, EKA 8, and EKA 20 were *Bacillus brevis* (EKA 4), *Bacillus badius* (EKA 8), and *Bacillus pantothenticus* (EKA20) with percent of probability 91.3% and 85.0%, 95.2%respectively.

Discussion

Anatomically, the digestive tract consists of crop, foregut, midgut, and hindgut (Cruden and Markovetz, 1987). Enzymatic digestion generally occurs in the midgut intestine which produces degradation enzymes (Bagde *et al.*, 2013). Besides being produced by midgut, enzymes are also produced by endosimbion microbes that live in the digestive tract. Each part of intestine has a different microbial community. The results of previous studies on the visualization of digestive tract microorganisms *Periplaneta americana* and *Shelfrodella lateral* use culture-dependent or culture-independent based method shows that the anterior colon has the highest density/ abundance (Bignell, 1976; Schauer *et al.*, 2012). Different cockroach diets greatly affect the diversity of bacteria. Omnivorous cockroaches *Periplaneta americana* has a higher endosymbiont diversity compared to wood-feeding cockroach *Cryptocercus punctulatus* (Colman *et al.*, 2012; Pérez-Cobas *et al.*, 2015).

Bacteria as providers a source of carbohydrate by increasing the efficiency of polymers such as lignin, hemicellulose and cellulose, xylan and pectin, can also contribute to the digestion of lipids and pro-

Table 3. Physiological character of EKA 4, EKA 8, and EKA 20

Characteristic	EKA 4	EKA 8	EKA 20
Oxidase	+	+	-
Motility	+	+	+
Nitrate	+	+	+
Lysine	-	-	-
Ornithine	-	-	-
H_2S	-	-	-
Glucose	-	-	-
Mannitol	-	-	-
Xylose	-	-	-
ONPG	+	-	+
Indole	-	-	-
Urease	-	-	-
VP	+	+	+
Citrate	-	-	-
TDA	-	-	-
Gelatin	+	+	+
Malonate	-	-	-
Inositol	-	-	-
Sorbitol	-	-	-
Rhamnose	-	-	-
Sucrose	-	-	-
Lactose	-	-	-
Arabinose	-	-	-
Adonitol	-	-	-
Raffinose	-	-	-
Salicin	-	-	-
Catalase	+	+	+
Arginine	**-**	**-**	**-**

teins (Douglas, 2009; Visotto *et al.,* 2009). The role of endosymbiont bacteria in the digestive tract, among others, produces hydrolytic enzymes that decompose organic matter. The ability of cockroach endosymbiont bacteria to produce amylase enzymes was triggered by decomposer animal food. Bacteria could be present in large quantities in the digestive tract along with amylase and bacteria were involved in product digestion (Anand *et al.,* 2010). This is also supported by Supriyatna and Ukit's research (2016) that the presence of some cellulolytic bacteria in the intestines of black soldier fly larvae helped the digestion process in organic matter. Endogenous cellulase was present in some insects and termites (Taggar, 2015).

Some microbes that play a role in the digestive track of *Pycnoscelus surinamensis* were the group of Planctomycetaceae, Candidatus, Proteobacteria, Sulfurospirillum, Actinomyces, Lactobacillales, Betaproteobacteria, Desulfovibrionaceae, Ruminococcaceae, Micrococcales, and Weissella (Richards *et al.,* 2017). This study found a group of rod-shaped Gram-positive spore forming bacteria endorsed by the Firmicutes group. This study confirm the results of previous studies conducted by Richards *et al.* (2017), using a culture-independent method namely Next-Generation Sequencing (NGS), found that the bacterial community of *Pycnoscelus surinamensis* was dominated by Bacteroidetes, Firmicutes and Proteobacteria. Predominantions of the Bacteroidetes and Firmicutes group were common in omnivorous groups including *Blatellagermanica*, *Shelfrodella lateralis* (Schauer *et al.,* 2012; Carrasco *et al.,* 2014; Perez-Cobas, 2015). Based on Correia *et al.,* (2018), Enterobacter found in intestinal track of *Pycnoscelus surinamensis*. Some species of the genus Enterobacter can produce cellulase enzyme (Sari *et al.,* 2017).

The results of the enzymatic test of the isolates shelter in the digestive tract of *Pycnoscelus surinamensis* showed that the eight isolates were also able to produce the digestive enzymes, amylase, protease, cellulase, and lipase. Cellulose-decomposing microorganisms become inseparable parts of the digestive tract microflora of cockroaches and other insects that eat material consisted of cellulose (Bagde *et al.,* 2013). Sharma *et al.* (2018), successfully isolated eight endosymbiont bacteria (14K, 16K, 22K, 24K, 28K, 29K, 30K, 31K) from the *Periplaneta americana* intestinal track. All of these isolates were known to be able to excrete protease and cellulase enzymes. Isolate 29K which was genetically similarly with *Bacillus* spp. wasable to excrete cellulase, protease, and keratinase. Cruden and Markovetz (1979), found in *Periplaneta americana* and *Euderes posticus* that there were a number of obligate anaerobic bacteria that can degrade carboxymethyl cellulose. In this study the enzymatic index method was used to compare the enzyme excretion produced by each bacterium. The enzymatic index method is a fast and simple way to select isolates that are most potential for enzyme excretion (Castro *et al.,* 2014). An index value above 1.0 is an indication of enzyme secretion (Carrim *et al.,* 2006). Based on research Florencio *et al.* (2012), strains that show an enzyme index higher than 1.50 were considered as potential enzyme producers. Exploration of bacteria that produce hydrolytic enzymes from the digestive tract of potential decomposers would then be continued.

Conclusion

Eighty percent of isolated bacteria from cockroach's gut have potency to produce hydrolytic enzymes; amylase, protease, cellulase, and lipase. The potential bacteria producing these four enzymes are EKA 4, EKA 8 and EKA 20.

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