

Characterization of entomopathogenic fungi as a natural biological control of American cockroaches (*Periplaneta americana*)

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Abstract. Rosa E, Ekowati CN, Handayani TT, Ikhsanudin A, Apriliani F, Arifyanto A. 2020. Characterization of entomopathogenic fungi as a natural biological control of American cockroaches (*Periplaneta americana*). *Biodiversitas* 21: 5276-5282. Eradicating cockroaches using chemical insecticides produces residues that contaminate the environment and promote insect resistance. Biological control has become an option for controlling cockroaches. The use of entomopathogenic fungi is one of them. The aim of this study was to obtain a fungal isolate that was effective at killing cockroaches. The result of entomopathogenic fungi isolation from cockroaches obtained 4 isolates, IK1, IK2, IK3 isolates were included in the *Aspergillus* genus while IK4 was *Penicillium*. Extracellular enzymes serve as screening materials for biocontrol candidates. IK1 and IK3 isolates showed higher chitinase activity than the other two isolates. The highest mortality of *Periplaneta americana* at nymphs stage was 60% achieved by the treatment of *Aspergillus* sp. IK3 with a concentration of 109 conidia/mL. In adult stage cockroach the mortality was increased to 86%.

Keywords: *Aspergillus*, biological control, cockroaches, entomopathogenic fungi, nymphs

INTRODUCTION

Cockroaches are vectors of disease. Their bodies have been shown to carry not only parasites (Atiokeng Tatang et al. 2017) but also a number of antibiotic-resistant pathogenic bacteria (Moges et al. 2016). They were found both indoors (kitchen and toilet) and outdoors (drainage and sewer). Environment condition and organic matter of these habitats were attracted to feed. Cockroach contact with a number of household items due to their mobility has increased the risk of pathogen transmission to humans (Yusof 2018).

Parasites and pathogenic bacteria carried by cockroaches cause diseases in humans such as toxocariasis, pneumonia, urinary tract infections, dysentery, and diarrhea. In people with asthma and rhinitis, cockroaches also cause allergic reactions. To minimize above diseases cockroach control is required properly. Control was carried out either mechanically, chemically or biologically. Mechanical control constrained by the mobility of cockroaches and their populations (Mongeau et al. 2014; Sanchez et al. 2015). Chemical control was a promising solution. Zahraei-Ramazani et al. (2018) reported that 90% of the American cockroach (*Periplaneta americana*) population has been successfully controlled, using chemical control. Unfortunately, this treatment also

threatened non-target insects and resulted in resistance vectors and produced residues that pollute the environment (Nicolopoulou-Stamati et al. 2016).

Entomopathogenic fungi, was unconventional applications (Litwin et al. 2020) of the biological control options in controlling cockroaches. It has several advantages such as affordable cost, high yield, and not interfere the environment with the residue (Mantzoukas and Eliopoulos 2020). EPF collected from agricultural soil samples (Robles-acosta et al. 2019), rhizosphere of maize plants (Nelly et al. 2019), and soils and *Ixodes scapularis* (Tuininga et al. 2009). Several studies reported that entomopathogenic fungi such as *Aspergillus* sp., *Geotrichum* sp., and *Penicillium* sp., have been used as bioinsecticides against house flies (*Musca domestica*) in larval stage (Septiana et al. 2019), *Mucor* sp., *Penicillium* sp., *Aspergillus* sp., as a control for adult mosquitoes (*Aedes aegypti*) (Supiyanto et al. 2019) and *Metharizium anisopliae* as a control for the German cockroach population (*Blattella germanica*) (Lopes et al. 2013). Entomopathogenic fungi information on *Periplaneta americana* is still limited. The aims of this study were to determine the enzymatic characteristics of entomopathogenic fungi and their effect on the number of deaths in nymphs and adult stage of *P. americana*.

MATERIALS AND METHODS

Isolation and identification of entomopathogenic fungi

Entomopathogenic fungi were isolated from the body parts of *P. americana* by a moist chamber method. The moist chamber method was carried out by immersing the body parts of the *P. americana* cockroach in 70% alcohol for 30 seconds and sterile distilled water twice consecutively for 2 minutes for surface sterilization. It placed on moistened tissue in a Petri dish. It incubated at 28°C for 3-5 days (Jain et al. 2020)

Entomopathogenic fungi that grown were cultured at Potato Dextrose Agar. They were purified and followed by morphological observation using a slide culture (72 hours) completed with the data of the mycelium, conidia, and conidia-producing bodies. The characteristics were compared with them (Barnet and Hunter 1998).

Colloidal chitin preparation

Chitin powder (5g) was weighed and dissolved in 80 mL of concentrated HCl. The mixture was homogenized using a magnetic stirrer for 30 minutes and allowed to stand at 4°C for 24 hours. The mixture was filtered using glass wool. The filtrate obtained was added with 40 mL of cold distilled water and then homogenized using a magnetic stirrer. The pH was adjusted with the addition of 12 N NaOH to close to 7. The mixture was centrifuged at 7.500 rpm for 15 minutes. The filtrate obtained was separated from the supernatant and added with cold distilled water. It was again centrifuged at 7500 rpm for 15 minutes. The filtrate obtained was colloidal chitin (Suryadi et al. 2016).

Entomopathogenic fungi chitinolytic activity

The chitinolytic ability of entomopathogenic fungi was tested using a mixture of PDA media with 1% colloidal chitin. The media was sterilized in an autoclave for 15 minutes. Media that has been sterile added 500 mg/L of chloramphenicol. The media was poured into sterile Petri dishes. Entomopathogenic fungi isolate was inoculated into the test medium using a needle loop, after the media had solidified. Chitinolytic activity was detected by the formation of a clear zone around the fungus colony. The clear zone was visualized by sprinkling 0.1% congo red and washed using 1 M NaCl (Suryadi et al. 2016).

Entomopathogenic fungi lipolytic and proteolytic activity

Lipolytic test was carried out by growing entomopathogenic fungi on PDA media with 1% olive oil, 0.04% methyl red, and 1.5% Tween 80. Proteolytic test conducted at PDA media supplemented with 1% skim milk that has been pasteurized at 60°C for 15 minutes. Lipolytic and proteolytic activity was detected by the formation of clear zones around the fungal colonies (Choudhary and Jain 2012).

Enzymatic index

The enzymatic index was determined using a modified formula from the research of Lechuga et al. (2016):

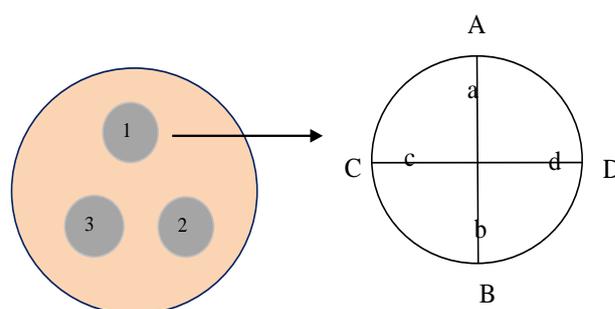


Figure 1. Measurement of colony diameter and clear zone diameter for enzymatic index determination

Note:
 AB, CD : Clear Zone Diameter
 ab, cd : Fungus Colony Diameter
 Average Clear Zone Diameter (Dz) : (AB+CD)/2
 Average Colony Diameter (Dk) : (ab+c)/2
 Average Total Clear Zone Diameter (RDz): (Dz1+ ... +Dzn)/n
 Average Total Colony Diameter (RDk) : (Dk1+ ... +Dkn)/n

The amount of the Enzymatic Index (IE) was determined based on the comparison of the average total diameter of the clear zone with the average total colony diameter with the following formula:

$$IE = (RDz - RDk)/RDk \quad (1)$$

Note* length in millimeter

Rearing nymphs of *Periplaneta americana*

Periplaneta americana nymphs were obtained from people's homes and reared in "rearing boxes" in the laboratory. They were fed bird feed pellets. Their egg sac was kept in a separate container until it hatches and then the nymphs that come out are separated into a new container. The test insects used were instar 3 nymphs (Arifah et al. 2016).

Fungal suspensions preparation

Sterile distilled water was poured into a test tube containing 14 days old entomopathogenic fungi. To the test tube 1% molasses and 0.5 mL Tween 80 were added. It was homogenized using a vortex mixer. Conidia density is calculated using a hemocytometer with a light microscope with a 400x magnification, with the following formula (Yunizar 2018):

$$C = (t \times 10^6)/(n \times 0.25) \quad (2)$$

Where:

C : The density of conidia per ml of solution
 t : The total number of conidia in the sample box was observed
 n : The number of sample boxes used
 0.25 : Small-scale sample box correction factor

The conidia suspension was further diluted at densities of 10^7 , 10^8 , and 10^9 conidia/mL.

Mortality test of entomopathogenic fungi toward *Periplaneta americana*

The cockroach nymph mortality was tested using dip insect method. It was modified from the (Gutierrez et al. 2015). Nymphs were immersed in a conidia solution with concentration whereas using the dip insect method with 3 mL conidia concentrations of 10^7 , 10^8 , and 10^9 . Conidial suspension composed of 7 days entomopathogenic fungi spores that diluted in water. It applied to each cockroach nymph and adult. This test was repeated 3 times and observed every 72, 96, 120 hours.

RESULTS AND DISCUSSION

Based on the results of macroscopic observations IK1, IK2, and IK3 isolates had colony characteristics with black color and different growth rates. Isolate IK4 was the smallest diameter in colony growth (Figure 2). In the three isolates of *Aspergillus* sp. had the same characteristics but there were differences in the size of the conidia (Table 1).

From the data obtained (Table 2), the highest mortality of *P. americana* nymphs was 60% in the treatment of *Aspergillus* sp. IK3 with a concentration of 10^9 conidia/mL was compared to control, whereas in the treatment of *Aspergillus* sp. The highest IK1 mortality of *P. americana* nymphs was 46% with a concentration of 10^9 conidia/mL. Based on the results of the ANOVA test ($\alpha = 5\%$), The type of entomopathogenic fungi isolates on the mortality percentage of *P. americana* nymphs did not show any significant differences. Although the mortality rate due to entomopathogenic fungi was low, the treatment of entomopathogenic fungi spores was proven to be able to kill cockroach nymphs (Figure 3).

Based on the results of the ANOVA test ($\alpha = 5\%$) (Table 4) that followed by Duncan double hose further test (Table 3), the type of entomopathogenic fungi isolates on the mortality percentage of *P. americana* nymphs was shown any significant differences. *Aspergillus* sp. 3 caused mortality to adult *P. americana* which was 86% higher than that of *Aspergillus* sp. 1 which was only 60%. The administration of entomopathogenic fungi spores was even more effective in killing adult cockroaches (Figure 4). This can be seen from the large percentage of deaths at spore density 10^9 when compared to the same density at the nymph stage.

Figure 5 informed that the highest chitinolytic index was produced by *Aspergillus* sp. 3 equal to 0.33 EI. Isolate *Aspergillus* sp. 3 was also an isolate that produced the largest clear zone diameter of 50 mm. The highest proteolytic index was produced by isolates *Penicillium* sp. equal to 0.50. However, the widest clear zone was produced by *Aspergillus* sp. 3 isolates, namely 34.7 mm,

larger than the clear zone of *Penicillium* sp. ie 15 mm (Figure 6). The highest lipolytic index was produced by isolate *Penicillium* sp. equal to 0.31. Unfortunately, the largest clear zone diameter was produced by *Aspergillus* sp., 2 isolates, which was 18 mm larger than the clear zone of *Penicillium* sp. by 8.5 mm.

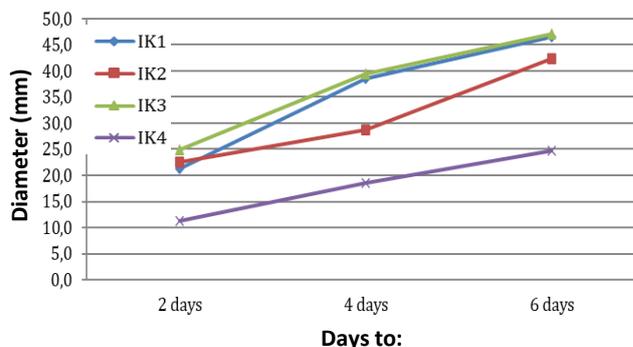


Figure 2. Colony growth of entomopathogenic fungi from cockroach (*Periplaneta americana*)



Figure 3. Morphology of *Periplaneta americana* nymphs after treatment (left) treatment of *Aspergillus* sp. 1, (right) treatment of *Aspergillus* sp. isolates 3



Figure 4. Morphology of *Periplaneta americana* adult after treatment (left) control, (middle) treatment of *Aspergillus* sp. 1, (right) treatment of *Aspergillus* sp. isolates. 3

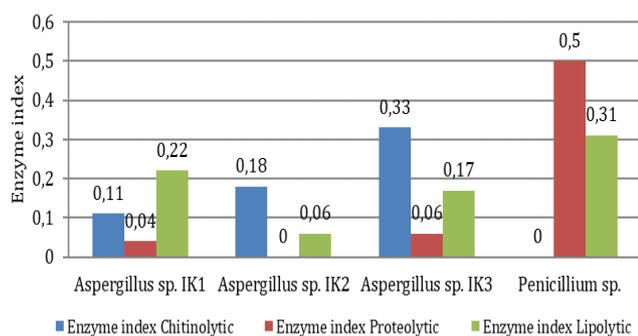


Figure 5. The performance of enzyme index of entomopathogenic fungi

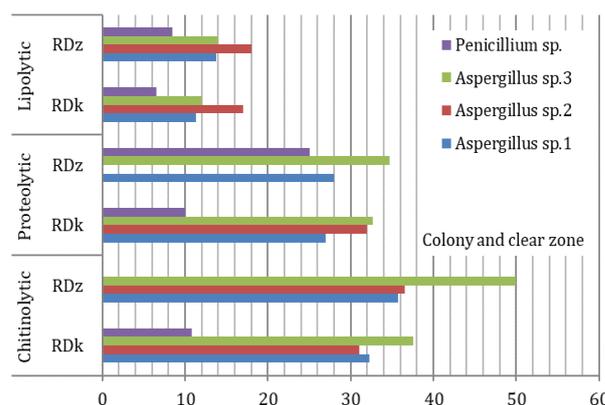


Figure 6. Colony and clear zone comparison of entomopathogenic fungi performance

Table 1. Morphology of entomopathogenic fungi from cockroach (*Periplaneta americana*)

Isolates	Morphology	Description
IK1		 Insulated hyphae, conidiophores upright and simple, there are leg cells, vesicles were semi-spherical, single filial and conidia size was 40 μm. It was presumed as <i>Aspergillus</i> sp.1
IK2		 Insulated hyphae, conidiophores upright and simple, there are leg cells, vesicles were semi-spherical, single filial and conidia size was 40 μm. It was presumed as <i>Aspergillus</i> sp.2
IK3		 Insulated hyphae, conidiophores upright and simple, there are leg cells, vesicles were semi-spherical, single filial and conidia size was 80 μm. It was presumed as <i>Aspergillus</i> sp.3
IK4		 Insulated hyphae, conidiophores erectly branched, absent in leg cells and vesicles, single filial, and conidia size was 40 μm. It was presumed as <i>Penicillium</i> sp.

Table 2. Mortality of *Periplaneta americana* nymph in EPF treatment

Isolates	Suspension concentration (conidia/mL)	<i>P. americana</i> nymph mortality				
		R1	R2	R3	A	%
<i>Aspergillus</i> sp. IK1	10 ⁷	1	2	3	2	40
	10 ⁸	1	1	1	1	20
	10 ⁹	2	4	1	2.3	46
<i>Aspergillus</i> sp. IK3	10 ⁷	1	1	2	1.3	26
	10 ⁸	1	1	2	1.3	26
	10 ⁹	2	3	4	3	60
Negative control		0	0	0	0	0
Vehicle control		0	0	0	0	0

Note: R1: 1st Repetitions, R2: 2nd Repetitions, R3: 3rd Repetitions, A: Average, %: Mortality percentage

Table 3. Mortality of *Periplaneta americana* adult in entomopathogenic fungi treatment

Isolates	Suspension concentration (conidia/mL)	<i>P. americana</i> nymph mortality				
		R1	R2	R3	A	%
<i>Aspergillus</i> sp. IK1	10 ⁷	2	3	2	2.3	46
	10 ⁸	2	2	3	2.3	46
	10 ⁹	3	3	3	3	60
<i>Aspergillus</i> sp. IK3	10 ⁷	3	3	4	3.3	66
	10 ⁸	4	5	3	4	80
	10 ⁹	4	4	5	4.3	86
Negative control		0	0	0	0	0
Vehicle control		0	0	0	0	0

Note: R1: 1st Repetitions, R2: 2nd Repetitions, R3: 3rd Repetitions, A: Average, %: Mortality percentage

Table 4. Analysis of Variance (ANOVA) Test ($\alpha = 5\%$)

		Sum of Squares	Df	Mean Square	F	Sig.
IK1	Between Groups	.889	2	.444	2.000	.216
	Within Groups	1.333	6	.222		
	Total	2.222	8			
IK3	Between Groups	1.556	2	.778	1.400	.317
	Within Groups	3.333	6	.556		
	Total	4.889	8			
NC	Between Groups	.000	2	.000		
	Within Groups	.000	6	.000		
	Total	.000	8			
VC	Between Groups	.000	2	.000		
	Within Groups	.000	6	.000		
	Total	.000	8			

Discussion

Based on the results of microscopic observations in Table 1, it was found 3 fungi *Aspergillus* sp. and 1 *Penicillium* sp. The data obtained for the IK1, IK2, IK3 isolates is in accordance with the opinion of Soesanto et al. (2020) which states that the *Aspergillus* sp., microscopically, it has a round conidium with a bluish-

green color, the head of the conidia (vesicle) is shaped like a club (clavate) or round, and becomes oval (columnar) with increasing age of the colony and has a conidia stalk (conidiophore). In addition, according to (Praja and Yudhana 2018) the fungus *Aspergillus* sp. have sterigmata appear to cover the upper half of the vesicles and have conidia with a serrated surface. The difference in the size of conidia (Table 1) caused by differences in gene regulation that regulate the formation of conidia through the conidia process so that gene expression is also different (Yu 2010).

IK4 isolate had different characteristics from the other three isolates. Based on the data obtained, IK4 has characteristics in accordance with the opinion of Visagie et al. (2014) that the fungal characteristics of the genus *Penicillium* sp. namely having hyphae with septa, conidia, sterigma, and conidia is caused by differences in gene regulation that regulate the formation of conidia through the conidia process so that gene expression is also different (Yu 2010).

The mortality test of entomopathogenic fungi used 2 isolates of fungi that had the highest chitinolytic index. In this case, the isolates used were *Aspergillus* sp. IK1 and *Aspergillus* sp. IK3. Veliz et al. (2017) emphasized that chitin was an essential component of insects and fungi exteriors. Degradation of external surfaces contains this polymer altered damage or even death of any pathogen. The clear zone formed shows the hydrolysis reaction of chitin polymer to N-acetyl D-glucosamine monomer by the chitinase enzyme produced by the fungus. The clear zone was seen more clearly after the addition of a solution of congo red (C₃₂H₂₂N₆O₆S₂Na₂) which binds to the chitin polymer in agar with β 1.4 N-acetyl-glucosamine bonds. Chitin polymer that has been hydrolyzed into N-acetyl-D-glucosamine monomers did not have β 1.4 N-acetyl-glucosamine bonds so that congo red did not bind strongly to these monomers which results in the formation of a clear zone around the colony. The clear zone will be seen more in contrast with the addition of 1 M NaCl which will dissolve congo red which is not strongly bonded, especially around fungal colonies (Schiavone et al. 2014).

Chitinases were particularly attractive to fungicidal, insecticidal, and nematocidal activities purposes (Berini et al. 2018). Chitin-degrading fungi including *Aspergillus*, *Mucor*, and *Mortierella* (Brzezinska et al. 2014). According to Utami and Ambarwati (2014) differences in the effectiveness of entomopathogenic fungi isolates in killing insects were influenced by several factors, including extracellular enzymes secreted by fungi to degrade insect bodies, the types of toxins produced, and conidia size. Based on the research that has been done, *Aspergillus* sp. 3 has a greater chitinolytic and proteolytic index compared to *Aspergillus* sp. 1 (Figure 4). This shows the ability of *Aspergillus* sp. 3 in secreting extracellular enzymes such as chitinase and protease is higher when compared to isolates of *Aspergillus* sp. 1. These enzymes are needed to penetrate the mechanical defenses of adult *P. americana* so that the entomopathogenic fungi can carry out further infection processes in the target insect's body.

Therefore, the mortality of *P. americana* adults was higher when treated using *Aspergillus* sp. 3 compared to isolates of *Aspergillus* sp.1.

This is supported by research conducted by Dhawan and Joshi (2017) which states that the activity of extracellular enzymes produced by entomopathogenic fungi is one of the determining factors for the level of virulence. The most important enzyme in this process was chitinase, which plays a role in degrading the chitin polymer contained in insect cuticles. Direct testing of third instar *Pieris brassicae* larvae experienced the highest mortality after being treated using the entomopathogenic fungi *Beauveria bassiana* which had the highest chitinase activity.

Another enzyme activity required to penetrate the host body is protease. This enzyme will break down the protein in the insect's body into amino acids which are used for the growth of entomopathogenic fungi. Based on research conducted by Perinotto et al. (2014) entomopathogenic fungi that have higher proteolytic activity have a higher virulence level as well.

Apart from chitinase and protease activity, lipase activity is also an important part of determining the virulence of entomopathogenic fungi. Lipase functions to hydrolyze ester bonds in lipoproteins, fats, and waxes on the inside of the insect integument so that it will make it easier for entomopathogenic fungi to penetrate. This is in accordance with research conducted by Dhawan and Joshi (2017), entomopathogenic fungi that have low lipase activity, are less effective in controlling the larvae of *Pieris brassicae*.

Aspergillus sp. was a fungus capable of producing mycotoxins such as ochratoxin A, B, and C, aflatoxins B1, B2, G1, G2, M1, and M2 (Martindah and Bahri 2016), and fumonisin B2 (Schuster et al. 2002). According to (Zain 2011) aflatoxins are immunosuppressive which can disrupt the immune system of adult *P. americana* which is attacked by entomopathogenic fungi. Meanwhile, ochratoxin works by disrupting the nervous system of the neurons and psychomotor speed so that adult *P. americana* has slower moving speed and reduced appetite which can lead to slow death (Ratnaseelan et al. 2018).

Another factor that affects the effectiveness of the entomopathogenic fungi is the density of conidia used in the test. Based on the research that has been done, the highest mortality was found in the treatment using isolates with conidia density of 10^9 conidia/mL for both types of entomopathogenic fungi. Meanwhile, the lowest mortality was found in the treatment using conidia density 10^7 and 10^8 conidia/mL for *Aspergillus* sp. 1 and 10^7 conidia/mL for *Aspergillus* sp. 3.

In the cockroach nymph stage test, the mortality rate was lower than that of adult cockroaches. Qian et al. (2010) proved that 6 week-old nymphs were more tolerant to dichlorvos and propoxur than the other ages tested, whereas both types of materials are chemical pesticides. The specific activity of acetylcholinesterase (AChE) took responsibility for this tolerance. Carbamate and organophosphate insecticides were like target sites for AChE. The inhibition of AChE by carbamate or

organophosphate insecticides occurs via a reversible complex formation followed by carbamylation or phosphorylation. AChE is a key enzyme in the transmission of nerve impulses, specifically in termination of cholinergic synaptic transmission in mammals and insects. The primary mechanism of acute toxicity of carbamate and organophosphate insecticides has been reported as its inhibition of AChE in the cholinergic synapse of the nervous system of the cockroach. The specific activity of AChE decreased significantly with increasing age. Thus, the application of entomopathogenic fungi to cockroaches as a biocontrol was influenced by the age of the target insects.

In conclusion, there were 4 entomopathogenic fungi isolates obtained in this study consisting of 3 isolates of *Aspergillus* sp and 1 *Penicillium* sp. Chitin-degrading enzymes were used as key characteristics of candidate biocontrol agents against *P. americana*. Further testing of biocontrol activity on cockroaches using *Aspergillus* strains IK1 and IK3 showed the mortality of *P. americana* in both the nymph and adult stages. This study showed that the entomopathogenic fungi obtained was more effective in controlling cockroaches in the adult stage than nymphs.

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