Crustaceous wastes as growth substrates for insect-pathogenic fungus *Metarhizium majus* UICC 295

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**Abstract.** The genus *Metarhizium* consists of a diverse group of species, which have a global distribution and a wide range of insect hosts. *Metarhizium majus* was reported to inflict 100% mortality on *Oryctes rhinoceros* beetle. Feeding activity of the *O. rhinoceros* beetle causes major crop loss in many coconut and palm oil plantations. This study investigated the use of crustaceous wastes and colloidal chitin as substrates for *Metarhizium majus* UICC 295 and the fungal virulence on *O. rhinoceros* larvae. Morphology of *M. majus* was observed on Sabouraud Dextrose Agar with Yeast Extract (S DAY) added with 5, 10, and 15 % (w/v) powder prepared from crab shell, green mussel shell, shrimp shell, or colloidal chitin and without addition as control. Fungal cultures in 10 % (w/v) crustaceous wastes or colloidal chitin were selected and virulence of *M. majus* UICC 295 was assayed by dripping the larvae with cell suspension. Untreated larvae were dripped with sterile distilled water and served as control. Mortality was recorded daily for 15 days. Fungal culture prepared from powder of colloidal chitin, green mussel shell and crab shell inflicted 100 % larval mortality in 10, 12, and 13 days, respectively, compared to fungal culture prepared from SDAY in 11 days.

**Keywords:** crustaceous waste; *Metarhizium majus*; *Oryctes rhinoceros*

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1. **Introduction**

The genus *Metarhizium* Sorokin is composed of anamorph entomopathogenic fungi and frequently parasitised a broad range of insect species, which are found in the tropics. Members of the genus *Metarhizium* have been used as biological control [1].

Species of *Oryctes* are susceptible only to all *Metarhizium* strains isolated from *Oryctes* species [2]. Cuticular chitin of the insects is covered with proteins and lipids. Thus, proteases and esterases are released first by the fungus followed by chitinases. The beginning of the host invasion by the fungus is penetration of the cuticle layer [2]. All entomopathogenic fungi are dependent on the production of cuticle-degrading enzymes (lipases, chitinases, proteases) to help penetrate the host cuticle [3]. The protease Prl de-repressed under starvation conditions and repressed in the presence of excess nutrients [4].
Nutrition influenced growth, sporulation and virulence of *Metarhizium anisopliae* on insect larvae. Inoculum produced on naturally infected arthropods usually is highly effective to susceptible hosts whereas that produced on artificial media can lose virulence [5]. Different amino acids stimulated particular stages of growth and sporulation, while a complex nitrogen source was required to optimize these processes [6].

There are several factors when developing inexpensive media for the mass production or bio-manufacturers of fungal biocontrol agent. Culture media must maximise spore yield and enhance qualities such as desiccation tolerance, and virulence [5].

Crustaceous wastes (i.e. shrimp shell, crab shell, and green mussel shell) are produced annually. These wastes may be utilized as the substrates for mass production of biocontrol agents. This study attempted to utilize crustaceous wastes and colloidal chitin for growth of *Metarhizium majus* UICC 295, and if the substrates affected the virulence of the fungus on *O. rhinoceros* larvae.

## 2. Materials and Methods

### 2.1 Microorganism and Oryctes rhinoceros cadaver

*Metarhizium majus* UICC 295 was isolated from infected *Oryctes rhinoceros* cadaver from Majalengka, West Java, and kept in Universitas Indonesia Culture Collection (UICC), Department of Biology, FMIPA Universitas Indonesia. The fungal isolate was identified by molecular method based on Internal transcribed spacer (ITS) region of 18S rDNA. The strain was maintained on Potato Dextrose Agar (PDA, Difco) containing 0.02 % (w/v) chloramphenicol (Wako). The infected cadaver was identified by Indonesian Institute of Sciences (LIPI).

### 2.2 Preparation of substrates

Crab shell, green mussel shell, and shrimp shell were cleaned under running water and washed by 70 % (v/v) alcohol. The shells were dried at room temperature, then crushed into powder using a blender (Phillips). After the shell powders were milled with 600 µm mesh, they were stored in a dry place at a room temperature. The shell powders were used as solid substrates without demineralization or deproteinization.

### 2.3 Preparation of fungus and inoculum

The strain was transferred to Sabouraud Dextrose Agar with Yeast Extract (SDAY) and SDAY added with 5, 10, and 15 % (w/v) crab shell, or green mussel shell, or shrimp shell, or colloidal chitin, on Petri dishes. The plates were incubated at 28 °C in the dark and observed for 15 days. Observation on conidia’s size, and hyphae’s width was carried out under light microscope (Carl Zeiss) (20 conidia and 20 hyphae). Inoculum for solid substrate was prepared from conidia/hyphae of 15-day old SDAY slant cultures incubated at 27 °C in the dark. The conidia/hyphae were scraped from slants and collected into sterile 0.05 % Triton X-100 to a final concentration of 10⁶ conidia/mL. Estimation of conidial concentration by using haemocytometer under light microscope (Carl Zeiss). The conidial concentration was diluted to make a final suspension of 10⁷ cell/mL with 0.05 % (w/v) Triton X-100. Conidial viability was determined by plating serial dilutions on SDAY added with 0.02 % chloramphenicol, three replicates each. The resulting colony-forming units (CFU) were counted after 4-7 days incubation at 28 °C.

### 2.4 Preparation of Oryctes rhinoceros larvae

*Oryctes rhinoceros* larvae were obtained from a cow ranch in Majalengka, West Java. The insect larvae were placed at room temperature (25 to 27 °C), high humidity (84 to 97 %) in the dark. Plastic small container (14.5×9.5×6 cm³) was used to rear each larva and every three days the larvae were fed with cow dung and palm tree waste. The larvae were pre-conditioned in the laboratory for one week. Test of *M. majus* UICC 295 virulence on the insect larvae was carried out according to Sari *et al.* [7].
The test was carried out on the larvae with *M. majus* UICC 295 prepared from SDAY and SDAY added with 10% shrimp shell powder. Each test was carried out on 30 larvae.

### 3. Results and Discussion

Observation on morphology of *Metarhizium majus* UICC 295 on SDAY and SDAY added with 5, 10, and 15 % crustacean wastes or colloidal chitin are shown in Table 1-4 and Figure 1-4. Addition of crustacean wastes and colloidal chitin in SDAY affected the fungal growth. In general, addition of crustacean wastes or colloidal chitin in SDAY increased the diameter, conidia and hyphae of the fungal colonies. Different sporulation was also observed on the media. Fungal colonies on SDAY showed zonation and lighter green colour compared to the fungal colonies on SDAY added with crustacean wastes or colloidal chitin. This observation was in line with a report from Bischoff *et al.* [1] which stated that strains of *Metarhizium* spp. showed colony pigmentation from white to yellow to greenish as the conidia matured. According to Ibrahim *et al.* [8], different culture media affected three strains of *M. anisopliae* by showing various pigmentation, size, and shape of conidia.

Better growth and full sporulation were shown by *Metarhizium majus* UICC 295 on SDAY added with 10% crustacean wastes or colloidal chitin were observed compared to 5% or 10% of the added substrates. Shah *et al.* [5] reported that spore production of *M. anisopliae* was highest in medium with CN ratio of 10:1. Fungal spores tend to be produced on carbon rich media, although the threshold varies with the species and the nature of the CN source. Chitin is a polysaccharide composed of β-1,4 N-acetyl-D-glucosamine units. It is distributed in nature, as a constituent of insect exoskeletons, shells of crustaceans and fungal cell walls [9].

#### Table 1. Observation of colony of *Metarhizium majus* UICC 295 on SDAY and SDAY added with 5 %, 10 % and 15 % crab shell powder, incubated at 28 °C in the dark for 14 days

| Observation of colony | SDAY | SDAY + 5 % crab shell powder | SDAY + 10 % crab shell powder | SDAY + 15 % crab shell powder |
|-----------------------|------|-------------------------------|-------------------------------|-------------------------------|
| Mean colony diameter (cm) | 2.09 | 2.89                          | 2.97                          | 2.87                          |
| Olive green           |      | Sea green                     | Sea green                     | Sea green                     |
| Mean conidia (width)  | 6.04±0.73 | 6.08±0.48                   | 6.54±0.71                    | 6.01±0.48                    |
| Mean conidia (length) | 23.76±2.11 | 23.78±1.17                   | 26.18±1.18                   | 23.95±1.18                   |
| Mean hyphal width     | 2.34±0.38 | 3.77±0.68                    | 4.59±0.93                    | 2.96±0.41                    |

#### Figure 1. Colony’s observation of *Metarhizium majus* UICC 295 on SDAY (a), SDAY added with 5 % crab shell powder (b), SDAY added with 10 % crab shell powder (c), and SDAY added with 15 % crab shell powder (d), incubated at 28 °C in the dark for 14 days
Table 2. Observation of colony of *Metarhizium majus* UICC 295 on SDAY and SDAY added with 5 %, 10 % and 15 % green mussel shell powder, incubated at 28 °C in the dark for 10 days

| Observation of colony | SDAY | SDAY + 5% green mussel shell powder | SDAY + 10% green mussel shell powder | SDAY + 15% green mussel shell powder |
|----------------------|------|-------------------------------------|-------------------------------------|-------------------------------------|
| Mean colony diameter (cm) | 2.08 | 2.62 | 2.67 | 2.46 |
| Mean conidia (width) | Olive green | Sea green | Sea green | Sea green |
| Mean conidia (length) | 6.04±0.73 | 5.41±0.40 | 5.83±0.58 | 5.13±0.43 |
| Mean hyphal width | 23.75±2.10 | 23.01±1.06 | 24.79±0.81 | 22.76±1.19 |

Figure 2. Colony’s observation of *Metarhizium majus* UICC 295 on SDAY (a), SDAY added with 5 % green mussel shell powder (b), SDAY added with 10 % green mussel shell powder (c), and SDAY added with 15 % green mussel shell powder (d), incubated at 28 °C in the dark for 10 days.

Table 3. Observation of colony of *Metarhizium majus* UICC 295 on SDAY and SDAY added with 5 %, 10 % and 15 % shrimp shell powder, incubated at 28 °C in the dark for 10 days

| Observation of colony | SDAY | SDAY + 5% shrimp shell powder | SDAY + 10% shrimp shell powder | SDAY + 15% shrimp shell powder |
|----------------------|------|-------------------------------|-------------------------------|-------------------------------|
| Mean colony diameter (cm) | 2.08 | 2.37 | 2.42 | 2.33 |
| Mean conidia (width) | Olive green | Sea green | Sea green | Sea green |
| Mean conidia (length) | 6.04 | 6.40 | 6.45 | 6.29 |
| Mean hyphal width | 23.75 | 24.09 | 24.68 | 24.69 |

Figure 3. Colony’s observation of *Metarhizium majus* UICC 295 on SDAY (a), SDAY added with 5 % shrimp shell powder (b), SDAY added with 10 % shrimp shell powder (c), and SDAY added with 15 % shrimp shell powder (d), incubated at 28 °C in the dark for 10 days.
Table 4. Observation of colony of *Metarhizium majus* UICC 295 on SDAY and SDAY added with 5 %, 10 % and 15 % colloidal chitin powder, incubated at 28 °C in the dark for 10 days

| Observation of colony | SDAY | SDAY + 5 % colloidal chitin powder | SDAY + 10 % colloidal chitin powder | SDAY + 15 % colloidal chitin powder |
|-----------------------|------|-----------------------------------|------------------------------------|-----------------------------------|
| Mean colony diameter (cm) | 2.08 | 3.35 | 3.38 | 2.88 |
| Mean conidia (width) | 6.04±0.73 | 5.8±0.90 | 6.57±0.64 | 5.76±0.73 |
| Mean conidia (length) | 23.76±2.11 | 23.72±1.89 | 24.38±2.24 | 22.17±2.46 |
| Mean hyphal width | 2.34±0.38 | 4.18±0.58 | 4.47±0.67 | 4.51±0.74 |

Figure 4. Colony’s observation of *Metarhizium majus* UICC 295 on SDAY (a), SDAY added with 5 % colloidal chitin powder (b), SDAY added with 10 % colloidal chitin powder (c), and SDAY added with 15 % colloidal chitin powder (d), incubated at 28 °C in the dark for 10 days

Exposure of *Metarhizium majus* UICC 295 conidia on *O. rhinoceros* larvae was shown on Figure 5 to Figure 8. Fungal culture prepared from powder of colloidal chitin, green mussel shell and crab shell inflicted 100 % larval mortality in 10, 12, and 13 days, respectively, compared to fungal culture prepared from SDAY in 11 days. In contrast, fungal culture prepared from shrimp shell powder caused fewer numbers of larval mortality compared to fungal culture prepared from SDAY. Hashim and Ibrahim [10] isolated *Metarhizium anisopliae* var. *majus* from *Oryctes rhinoceros*. 100 % larval mortality of *Crocidolomia binnotalis* was observed at 2×10⁷ conidia mL⁻¹ for *Metarhizium anisopliae* var. *majus*.

Figure 5. Percentage of mortality of insect larvae after exposure to conidia of *M. majus* UICC 295 prepared from SDAY and SDAY added with 10 % colloidal chitin powder.
Figure 6. Percentage of mortality of insect larvae after exposure to conidia of *M. majus* UICC 295 prepared from SDAY and SDAY added with 10% green mussel shell powder.

Figure 7. Percentage of mortality of insect larvae after exposure to conidia of *M. majus* UICC 295 prepared from SDAY and SDAY added with 10% crab shell powder.

Figure 8. Percentage of mortality of insect larvae after exposure to conidia of *M. majus* UICC 295 prepared from SDAY and SDAY added with 10% shrimp shell powder.
According to Shah et al. [5] starvation exhaust endogenous reserves in conidia and would induce Prl and other virulent determinants. Prl is a protease and a virulent determinant which is induced by virulent cuticle. Conidia from insects with mycoses were aggressive since they had low carbon content (ca. 30-50 % lower than conidia from artificial media) and subsequently fewer endogenous reserves (e.g. glycogen, lipid). Wang et al. [4] reported that the virulence of M. anisopliae was influenced by several factors, i.e. the ability of conidia to adhere to the insect cuticle and the culture media for production of conidia.

4. Conclusion
This study showed that crustaceous wastes and colloidal chitin in the media could be used as substrates for M. majus UICC 295. These carbon sources provided nutrients for the fungus. Better growth and full sporulation were shown by M. majus UICC 295 on SDAY added with 10 % crustaceous wastes or colloidal chitin. Metarhizium majus UICC 295 was consistently virulent on O. rhinoceros larvae irrespective of the different substrates in the media. Further studies are required to examine the virulent stability of the fungal strain growing in crustaceous wastes after short-term storage.

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