

Diversity and characterization of entomopathogenic fungi from rhizosphere of maize plants as potential biological control agents

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Abstract. Nelly N, Syahrawati MY, Hamid H, Habazar T, Gusnia DN. 2019. Diversity and characterization of entomopathogenic fungi from rhizosphere of maize plants as potential biological control agents. *Biodiversitas* 20: 1435-1441. The diversity and characters of entomopathogenic fungi in soil are influenced by cultivation techniques. This study aims at determining the characters and pathogenicity of entomopathogenic fungi in maize plant. Materials were extracted from maize plant rhizosphere at different planting system (monoculture, polyculture, and monoculture of corn on formerly oil palm plantation or replanting) in Nagari Koto Baru, Luhak Nan Duo Subdistrict, West Pasaman District, and West Sumatra Province. Insect bait and series dilution were used to carry out the isolation, while *Tenebrio molitor* larvae were used for the pathogenicity test. Parameters observed include macroscopic and microscopic characteristics, and pathogenicity of entomopathogenic fungi. The result shows that there are three types of entomopathogenic fungi with various features. After identification, it was known that *Aspergillus* and *Metarhizium* were obtained from mono, polyculture and by replanting crops, while *Beauveria* was found from the rhizosphere of corn grown in polyculture with pathogenicity identified as one of the high causes of death to larvae *T. molitor* with LT50 ranging from 48.03-48.48 hours.

Keywords: Characterisation, diversity, entomopathogenic fungi, rhizosphere

INTRODUCTION

Pests and diseases affect the productivity of corn plants. And the main pests attack corn are corn borer (*Helicoverpa armigera*), armyworm (*Spodoptera litura*) and grasshopper (*Valanga nigricornis*) (Adnan 2011). Others such as *S. pacificus*, are found in field and spread in several regions in West Sumatra, (Nelly et al. 2017). The intensity of damages developed on maize is usually between 26-50% and weighs from 51-75%, owing to attacks from armyworms, grasshoppers and cob borer. To overcome these damages, adequate control efforts are required (Fattah and Hamka 2008).

Generally, corn pest is controlled by using resistant varieties, technical culture or synthetic insecticides. However, this has not been effective because these pests have short life cycles and high adaptability (Bakhri 2007 and Patty 2012). Furthermore, the continuous use of synthetic insecticides can have some negative effects on the environment. It could lead to resistance, resurgence, residue, and death (Sofia 2001). To overcome this problem, other environmentally friendly biological control techniques should be utilized (Baliadi et al. 2008). Biological control for pests consists of predators, parasitoid and *entomopathogen* (Habazar and Yaharwandi 2006).

Entomopathogenic is a microorganism capable of treating diseases in insects. It comprises of fungi, bacteria, viruses, nematodes, and protozoa (Habazar and Yaharwandi 2006). Several species reported to be effective in controlling insect pests are *Beauveria bassiana*, *Metarhizium anisopliae*, *Nomuraea rileyi*, *Paecilomyces*

fumosoroseus, *Aspergillus parasiticus* and *Verticillium lecanii* (Prayogo 2006). *B. bassiana* and *M. anisopliae* effectively control green ladybugs (*Nezara viridula*) 96.40% (Suprayogi et al. 2015). *N. rileyi* effectively controls *H. armigera* 36-85.56% (Indrayani 2013). The *P. fumosoroseus* Ultra Low Volume (ULV) formula with an additional 15% corn oil effectively controls *Bemisia tabaci* (Kamalin 2011). *A. parasiticus* is effective in controlling *S. litura* 65% (Prayogo 2006). *V. lecanii* with conidia 108 / ml density effectively control green leafhopper (*Nephotettix virescens*) 50% (Ladja 2009).

Entomopathogenic source comes from plants such as endophytic fungi, infected insects, and from soil or rhizosphere. Entomopathogenic fungi are often found around the rhizosphere of plants in soil, because its availability is higher (Carlile et al. 2001). *Beauveria*, *Metarhizium*, *Nomuraea*, *Paecilomyces*, *Fusarium*, and *Aschersonia* are found in cabbage and vegetable plants (Nuraida and Hasyim 2009) *Metarhizium* (Trizellia et al. 2016). *Metarhizium* sp., *Beauveria* sp., *Penicillium* sp. and *Aspergillus* sp. are found in rhizosphere of corn in Lampung region (Semenguk 2016).

The presence of *entomopathogenic* fungi in soil is influenced by several factors, such as the content of soil water, organic matter and compounds (Carlile et al. 2001). Furthermore, the diversity of *entomopathogenic* fungi is also influenced by the sea level's cultivation and altitude (Trizellia et al. 2015) as well as the types of protective plants affected by microorganisms (Hamdani 2009). Type of planting pattern in the field that is thought to affect the presence of fungi, namely polyculture, monoculture and

replanting. Polyculture is a form of agriculture in which more than one species is grown at the same time and place in imitation of the diversity of natural ecosystems (Chrispeels and Sadava 1994). Monoculture, in which only members of one plant species are cultivated and replanting is replanting plants that do not produce. The diversity of entomopathogenic fungi in polyculture cropping is higher in the highlands. It consists of 9 isolates, while the monoculture cropping pattern consists of 7 (Trizelia et al. 2015).

Entomopathogenic indigenous fungi are said to be more effective for pest control. According to Khasanah (2008), *B. bassiana* local strain concentration of 0.6 mg / l water in sweet corn plants is effectively used in controlling corn borer. It is often planted mono and polyculture or on oil palm plantations being replanted. Information about indigenous entomopathogenic fungi related to these cultivation techniques has not been widely reported. As a result, this research was carried out with the aim of obtaining entomopathogenic fungi from rhizosphere in monoculture, polyculture, and oil palm replanting.

MATERIALS AND METHODS

Study area

This research was conducted and implemented from November 2017 to May 2018 at the Biological Control laboratory, Faculty of Agriculture, Andalas University, Padang, West Sumatra, Indonesia with sampling carried out at Jorong Ophir and Giri Maju, Nagari Koto Baru, Luhak Nan Duo Sub-district, and Pasaman Barat District, West Sumatra, Indonesia.

Material and tools

Materials and the tools used were maize plant rhizosphere, medium *Sabaoraud Dextrose Agar with Yeast Extract* (SDAY), *Potato Dextrose Agar* (PDA), hemocytometer Improved Neubauer nesco (vol. 0,0002 mm²), etc.

Sampling of rhizosphere soil from maize plants and isolation of entomopathogen fungi

Three samples soil were taken and extracted from monoculture, polyculture, and palm replanting sites. The soil is taken by digging around the rhizosphere with a depth of 10-20 cm and a stem distance of 3 cm. About 1 kg of each plant is put into a plastic bag and taken to the laboratory. Rhizosphere soil is also extracted from the root, by removing the corn plants. The fungus isolation was carried out by insect bait and serial dilution method.

Insect bait

According to Trizelia et al. (2015), the insect bait method is achieved using *T. molitor* larvae. These are maintained in plastic boxes measuring 25 cm x 15 cm and fed 100 g of larvae daily. Soil around the rhizosphere from 5 points is combined and sieved with a 60 mesh. After sieving, the sample is divided into 5 and placed in a plastic box measuring 10 x 15 cm. The soil is moistened with 100

ml of distilled water and 10 *T. molitor* larvae, with 2 cm of its length put into skin. *T. molitor* larvae are covered with a layer of soil and observed every day until they die. The fungus attacked by fungi is cultured using the moist chamber method. Larvae is soaked with aquadest, and 70% Alcohol at an interval of one minute respectively to sterile the surface. The sterilized larvae are inserted into a petri dish containing moist tissue and incubated until the fungus grows at room temperature. Entomopathogenic fungi that grow are cultured on SDAY media, until ascertained pure using a microscope (Watanabe (2002); Bannett and Hunter (1972).

Serial dilution

The serial dilution technique (series dilution) refers to Trizelia et al. (2015) techniques which has been modified as follows: 10 g of plant roots are taken and homogenized in 100 ml of sterile distilled water for 2 minutes, 1 ml of suspension is put into the test tube containing 9ml distilled water and homogenized for 2 minutes. 1ml of the suspended dilution was transferred into a container containing PDA and incubated at room temperature until the growing mushroom filled the Petri dish. PDA mediums are observed every day. Each colony that grows and shows different fungal characteristics is re-isolated in the cuprion until pure culture is obtained. Pure fungus culture was tested by entering 10 *T. molitor* larvae that replaced the skin, and observed for 7 days. Fungi that can infect *T. molitor* larvae are re-cultured on SDAY media.

Identification of entomopathogenic fungi

The isolates of the entomopathogenic fungi were identified as macroscopically and microscopically. The macroscopic figure was observed in color and shape with the microscopic fungus identified using a microscope by observing the conidiophores branching and conidia. The identification results were compared with Watanabe (2002) and Bannett (1972).

Pathogenicity test of entomopathogenic fungi on larva *Tenebrio molitor*

Pathogenicity is the fungus capable of infecting *T. molitor* larvae was tested. The fungus is isolating every 10 ml for 24 hours. Larvae fed and fungus was transferred into a 5cm plastic tube. Larvae *T. molitor* were observed every 24 hours for 10 days.

Identification of entomopathogenic fungi

The fungus was identified at the genus level by observing macroscopic (color, colony shape, growth rate) and microscopic (conidiophores branching, conidia form, conidia density, and sprout power). Observation methods are as follows:

Fungi colony growth rate: The entomopathogenic fungus growth was measured by the size of the colony using millimeter paper on the second day until the fungus-filled the Petri dish.

Conidia density of entomopathogenic Fungi: Conidia density was calculated using the dilution method. The conidia of the fungus were suspended with 10 ml of

aquades in Petri dish containing pure culture. This is known as base suspension. Next, it is diluted to 10^{-3} and 1 ml of the suspended distillate taken with a micropipette and placed on a hemocytometer and covered with glass. The hemocytometer was placed under a microscope and number of conidia counted. Observations were carried out with low magnification. To calculate the density of conidia/ml, the following formula is used:

$$\text{Conidia density} = \frac{\text{The total number of conidia in the box observed}}{\text{Number of boxes observed}} \times 4 \times 10^6 \times P$$

Where:

P = large dilution

Conidial sprout: The fungus is suspended with 10 ml of aquades and homogenized with vortex. A drop is suspended on a glass object covered and placed in a Petridish containing moist filter paper. The suspension was incubated for 18 hours at room temperature after which conidia were germinated using a 40x magnification microscope. The percentage of sprouts was calculated from 50 conidia which are said to germinate when the length of the tube exceeds its diameter.

Pathogenicity of entomopathogenic fungi

Incubation period: The incubation period was observed to determine the time needed by the fungus to cause symptoms in *T. molitor* larvae.

Mortality of *Tenebrio molitor* larvae per day: Each day, the mortality rate of *T. molitor* larvae was calculated from the first until the 10th day of observation.

Total mortality of *Tenebrio molitor* larvae: Larval mortality rate was calculated based on the number of *T. molitor* larvae that died 10 days after treatment. Calculations are carried out every 24-hour interval using the formula:

$$M = \frac{n}{N} \times 100\%$$

note:

M = Larval mortality

n = Number of dead larvae

N = Number of larvae observed

Lethal Time (LT): The LT is calculated from the percentage of test larvae deaths. LT50 is the time point at which mortality of inoculated hosts (larvae) is 50%. To determine the value of LT50 used probit analysis.

Entomopathogenic fungus sporulation in *T. molitor*: Fungus sporulation was observed by calculating *T. molitor* larvae infected with entomopathogenic from the first till the 7th day after treatment with a 24-hour interval using the formula:

$$S = \frac{n}{N} \times 100\%$$

Where:

S = Sporulation of entomopathogenic fungi

n = Number of sporulating larvae

N = Number of larvae observed

RESULTS AND DISCUSSION

Identification of entomopathogenic isolates

Three entomopathogenic fungi with different features were found in the isolates with the results compared to Watanabe (2002) and Bannett and Hunter (1972). The fungi obtained were *Aspergillus*, *Metarhizium*, and *Beauveria* with the macroscopic and microscopic morphological observations shown in Table 1.

Amongst the three entomopathogenic fungi obtained, *Aspergillus* and *Metarhizium* were found in monoculture, polyculture and replanting soils, while *Beauveria* was only found in polyculture fields. The observation of incubation period, growth rate, germination and conidial density of each fungus isolate can be seen in Table 2.

From Table 2 it can be seen that incubation period, the growth rate, germination and conidial density of all isolates were not different based on the analysis of variance (ANOVA) ($P > 0.05$). The incubation period of all isolates is 3 days with symptoms arising a day after the larvae's death. Colony growth rates are also not too different ranging from 23-24 cm². While the highest germination rate was *Metarhizium* isolate which was 81%, with conidia density of 18×10^7 .

Pathogenicity of entomopathogenic fungi

The observation results of larval deaths due to entomopathogenic fungi can be seen after 24 hours. On the 5th and 7th day after death, sporulation was seen. *T. molitor* larvae attacked by entomopathogenic species of *Aspergillus* showed a yellowish color, *Metarhizium* greenish while that of *Beauveria* was white (Figure 1).

Dead larvae infected with entomopathogenic fungi are characterized by dry, stiff and color changes. After a day visible hyphae are seen growing around the region. The number of hyphae that grows continues to grow in accordance with increasing time. In the end, the entire body of the larva will be enveloped by entomopathogenic fungal hyphae.

Table 2. Incubation period, growth rate, germination and conidia density of entomopathogenic fungi from corn rhizosphere

Isolate	Incubation period (days)	Growth rate (cm ²)	Germination (%)	Conidia density
<i>Aspergillus</i>	3	23.32	64	3.65×10^7
<i>Metarhizium</i>	3	24.65	81	1.8×10^7
<i>Beauveria</i>	3	23.47	73	1.6×10^7

Table 1. Macroscopic and microscopic entomopathogenic fungi from corn rhizosphere

Isolates	Morphology		Source of isolates (soil)
	Macroscopic	Microscopic	
<i>Aspergillus</i>	Colony color: yellowish green with white edges, Average colony size 23.32 cm ²	Hyphae: septa, Conidia: round, density: 3.65x10 ⁷	Monoculture Polyculture Replanting
<i>Metarhizium</i>	Colony color: White, yellowish over time will turn green. Average colony size 24,65 cm ²	Hyphae: septa. Conidia: round cylindrical, density 1.8x10 ⁷	Monoculture Polyculture Replanting
<i>Beauveria</i>	Colony color: White eventually turns yellow. Average colony size 23.47 cm ²	Hyphae: no septa conidia: round, density 1.6x10 ⁷	Polyculture

**Figure 1.** Larvae infected by *Aspergillus* sp. (A), *Metarhizium* (B), and *Beauveria* (C) on the 5th day after death

Mortality of *Tenebrio molitor* larvae

The average mortality rate of larvae by *Metarizium* and *Beauveria* was same with a 100% rate after treatment on day 3. In contrast to the test larvae applied with fungi, *Aspergillus* sp. had a mortality value of 18.33% for monoculture and polyculture, while isolates from replanting plants were 12.67%. The LT50 *Metarizium* fungus is isolated from maize plantations with cultivation or polyculture, replanting, and monoculture for 49.032 hours, 48.912 hours and 48.48 hours respectively. LT50 *Beauveria* fungus had duration of 49.368 hours while Fungus *Aspergillus* had a polyculture, monoculture, and replanting of 55.27 days, 37.20 days and 30.23 days (Table 3). It is estimated that *Aspergillus* isolates are entomopathogenic, but are not effectively used as control agents.

If the observed larval mortality rate is determined with different entomopathogenic fungi, variety of developments will be recognized. Similarly, the environmental conditions of the plant originating from different isolates, such as polyculture, monoculture, and replanting of oil palm plants, resulted in differences. *Aspergillus* fungi with different isolates (mono, polyculture and replanting) were then applied to *Tenebrio*, showing an increase in mortality starting after day 2 (Figure 2).

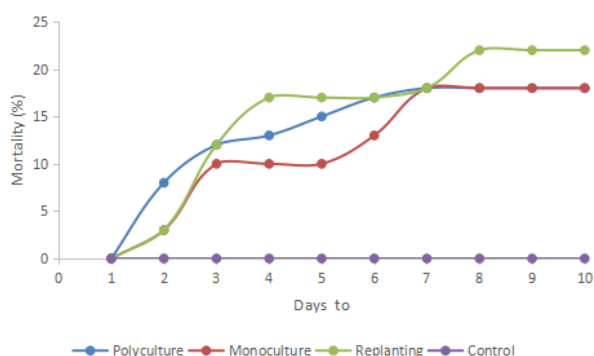


Figure 2. Mortality of *Tenebrio molitor* larvae per day after treatment of *Aspergillus* sp.

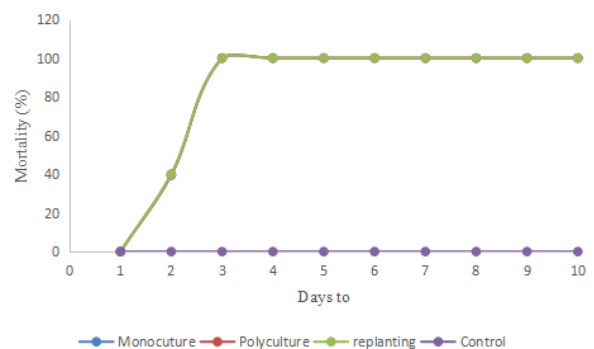


Figure 3. Mortality of *Tenebrio molitor* larvae per day after treatment with *Metarizium*

Larvae death due to *Aspergillus* sp. infection, commenced on the second day after treatment with different isolates from the plant origin. Isolates from monoculture land mortality were 3%, while that of day 3 was 10%. The number of new larvae deaths increased on the 6th and 7th day to 13% and 18% respectively. This percentage was steady until the 10th day of observation. Similarly, isolates from polyculture and replanting fields increased until the 10th day.

The result was obtained with the application of *Metarizium* and *Beauveria* fungi. Death occurs after the second day of application, and increased until the third to 10th day with 100% mortality rate (Figures 3 and 4).

Table 3. Mortality rates and LT50 *Tenebrio molitor* larvae after treatment of entomopathogenic fungi

Isolate	Land	Mortality (%)	LT50 (days)
<i>Aspergillus</i>	Polyculture	18.33	55.27
	Monoculture	18.33	37.20
	Replanting	21.67	30.23
<i>Metarhizium</i>	Polyculture	100	2.04
	Monoculture	100	2.02
	Replanting	100	2.04
<i>Beauveria</i>	Polyculture	100	2.06

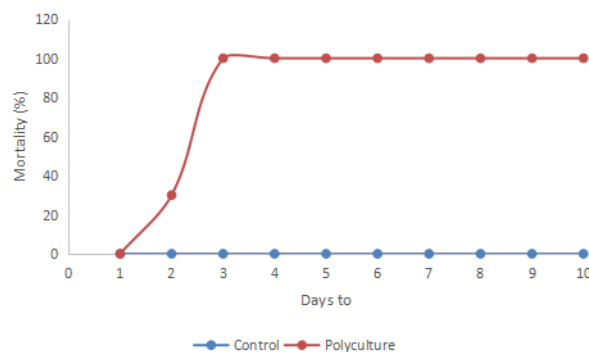


Figure 4. Mortality of *Tenebrio molitor* larvae per day after treatment with *Beauveria*

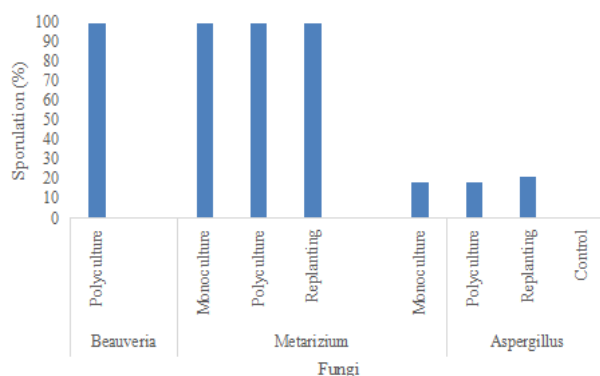


Figure 5. Average sporulation of entomopathogenic fungi in *Tenebrio molitor* larvae after treatment with isolate from different fields

Larvae death caused by *Metarizium* fungi occurred on the second day. Mortality *T. molitor* larvae on the second day by isolates different fields; 42% monoculture, 35% polyculture, and 37% replanting. On the third day, it increased to 100%. That of *Beauveria* occurred on the second day, by 30% and the third day it increased to 100% (Figure 4).

Entomopathogenic fungus sporulation in *T. molitor*

The highest sporulation rate of entomopathogenic fungi by *Metarizium* and *Beauveria* is 100% and the lowest *Aspergillus* is 18.33-21.67% (Figure 5).

The average sporulation of *Metarizium* and *Beauveria* fungi was the same, (100%) and different from the fungus *Aspergillus*. which has a sporulation value of only 18.33-21.67%. So the effectiveness of *Aspergillus* is much lower than *Metarizium* and *Beauveria*. However, when compared with controls, entomopathogenic fungi differ so much that the entomopathogenic fungi obtained are able to kill *T. molitor* larvae.

Discussion

Three entomopathogenic isolates were found in the rhizosphere of maize plants taken from different cultivated fields, namely mono and polyculture as well as on replanted palm oil fields. Entomopathogenic fungi can be found in the rhizosphere, soil, plants or in infected insects (Wilyus and Schue 2015). Its presence is influenced by several factors such as site height, protective plant species and cultivation techniques (Hamdani 2009; Trizelia et al. 2015). One cultural method that influences the diversity of entomopathogenic fungi is the planting system, such as monoculture and polyculture. Corn in some areas is also planted on replanted land or oil palm.

The advantage of polyculture planting systems is the diversity of living things and high organic matter. In addition, it affects soil temperature, with high moisture because its surface is covered by a leaf canopy (Nurindah 2006). The content of organic matter and soil moisture is directly proportional to the population of microorganisms. The higher organic content of matter causes the greater diversity (Wicaksono et al. 2015). Carlile et al. (2001) reported that the diversity of entomopathogenic fungi was influenced by organic matter, soil water content, temperature, and soil microorganism population. This is directly proportional to the results of this study which obtained data on the diversity of entomopathogenic fungi in polyculture higher than monoculture and replanting. Monoculture and replanting, comprises of 2 types of entomopathogenic fungi, *Aspergillus* and *Metarizium* while polycultural land has 3 types of entomopathogenic fungi, namely *Aspergillus*, *Metarizium* and *Beauveria*. The low diversity of entomopathogenic fungi on monoculture land is caused by the diversity of vegetation and low organic matter, while on replanting land it is as a result of the use of herbicides. Emalinda et al. (2003) reported that the use of herbicides on the soil affects the population of microorganisms in the soil, improper use will reduce the population of microorganisms because the active

ingredients contained can kill these organisms, thereby, affecting plant growth.

The effectiveness of entomopathogenic fungi is known from the mortality of test larvae. The higher the mortality, the more effective the fungus is used for control. The sporulation determines whether the larvae die due to the test fungus or not. From this study, the value of mortality and sporulation values were high by the *Metarizium* and *Beauveria* fungus, i.e. 100% while the *Aspergillus* has a mortality value of 18.33% for isolates from monoculture and polyculture fields and 12.67% replanting with sporulation values ranging from 10-40%. This result is the same as the value of LT50 obtained with pollutant, replanting, and monoculture extracted in 2.043, 2.038 and 2.02 days. LT50 *Beauveria* is 2.057. LT50 *Aspergillus* is comprising of polyculture 55.27 days, monoculture 37.20 days and replanting 30.23 days. *Aspergillus* isolates from any field are not effective, because of the longtime lethal. Whereas *Beauveria* and *Metarizium* isolates have a short value of LT50.

Differences in mortality values, sporulation values, and LT50 were obtained from *Metarizium* and *Beauveria* fungi with *Aspergillus*. It is related to the effectiveness of entomopathogenic fungus. According to the results reported by Erlina (2016), the value of LT50 is used by *B. bassiana* fungus to control *Etiella zinckenella* faster than the treatment of fungi *Aspergillus* sp. in 7.10 days while in *Aspergillus* sp. 18.30 days. This is similar to the results of this study which states that the ability of *Aspergillus* fungus isolated to determine the cause of death was only tested at 18-21%. It can be said that this fungus is less effective compared to the *Metarizium* and *Beauveria* fungi.

Based on the results and discussion, three types of entomopathogenic fungi with different characters and fields were analyzed. After being identified, *Aspergillus* and *Metarizium* from monoculture, polyculture and replanting land were obtained. While, *Beauveria* was found only in polyculture. The pathogenicity of *Metarizium* and *Beauveria* is capable of causing deaths of up to 100% of larvae of *T. molitor* with LT50 ranged from 48.03-48.48 hours.

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