Beauveria bassiana Growth and Development in Various Liquid Media

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Beauveria bassiana Growth and Development in Various Liquid Media

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Abstract. *Beauveria bassiana* is entomopathogenic fungi that can be cultivated on solid or liquid media. The cultivation of *B. bassiana* on cracked corn medium leaves unwanted waste. Therefore, cultivating *B. bassiana* on liquid media is expected to shorten the cultivation time and leave no waste. The purpose of the research was to analyze the physical characteristics of the B. *bassiana* colony, nutritional changes of the liquid media, and the pH level changes of the liquid media. This research used an experimental complete randomized design with six treatments and 4 times repetitions. The main phases of this research were the subculture of *B. bassiana* isolate; the preparation of liquid media consisting of distilled water, Potato Dextrose Broth (PDB), Potato Sugar extract (PSE), PSE+NPK 1%, PSE+coconut water, PSE+NPK 1%+coconut water each for 150 ml; *B. bassisana* inoculation into the liquid media; incubation process for 30 days; and measurement of fungal blastospore density, medium pH, and carbohydrate, protein, and lipid content. The results obtained from the morphological observation of *B.bassiana* on liquid media showed that the colonies are white, growing on the surface of the media with a powdery texture. There are some differences in the nutrient content of the media after 30 days of *B. Bassiana* incubation. After the incubation, there is an increase in carbohydrates and a decrease in protein and lipid content, as well as an increase in the pH level of the media.

Key words: *Beauveria bassiana;* liquid media; Potato Sugar Extract (PSE)

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INTRODUCTION

One of the environmentally friendly pest control is by using biological control agents. Biological control agents can be obtained from viruses, bacteria, or fungi. Biological pest control has several advantages since it causes no toxicity to the environment and other living organisms does not create any resistance, is selective against target pests, and causes no harmful effect (Sunarno, 2012). Biological agents from the fungal group are including *Metarhizium* sp, *Trichoderma* sp, and *Beauveria bassiana*. *B. bassiana* is a parasitic fungus that can be used as an insect pest control agent or is known as an entomopathogen (Subasinghe at al., 2013; Zulfiana et al., 2020). Studies about the general physical characteristics and the pathogenicity of *B. bassiana* against the insect's host have reached a massive level of research in the last decade.

The advantages of *B. bassiana* are its ability to inhabit the leaf surface of several plant varieties, to inhabit the soil as a saprophyte (Greenfield et al., 2016; Affandi et al., 2013), to live as an endophytic in some plants (Mcguire et al., 2020), and to infect 707 insect hosts from 521 genera, 149 family, and 15 ordos (Mwamburi, 2020). Some research stated that *B. bassiana* can control some species such as *Spodoptera litura* (Indriyanti et al., 2017a) and *Helopeltis* sp (Indriyanti et al., 2017b). Another predominance of *B. bassiana* is the ability to adapt to 25-30°C environmental temperature (Alali et al., 2019).

One of the institutions that produce the biological control agent, especially *B. bassiana* is The Center for protection of food crops, horticulture, and plantations (BPTPHP), Central Java Province in Salatiga. This center used cracked corn as a solid medium for the fungi. This kind of medium can only be used once. After the conidia

are harvested, the medium (cracked corn) will become waste or product that has not been utilized commercially. Therefore, another alternative is needed to breed *B.bassiana* with other media, including liquid media. Another weakness of this solid medium is the low conidial density obtained by only 7.8 X 10^5 (Pertiwi, 2016) It is lower than a standard from the Indonesia Ministry of Agriculture with $1x10^6$ conidial density. Alternative method is needed for *B. bassiana* cultivation using a liquid medium since it provides some advantages such as short cultivation time, no significant difference in the quality of the culture from one batch to another, and relatively easy to measure the pH, temperature, and oxygen levels (Mascarin, 2018).

Nutritional criteria needed for *B. bassiana* cultivation medium are rich in karbon, hidrogen, nitrogen, sulphur, and phosphorus (Latifian et al., 2013). However, in order to support the optimum growth indicated by high number of blastospore density and viability, it is important to provide addition supplementations. The potential supplementations are NPK (nitrogen, phosphate and potassium) fertilizer and coconut water. NPK fertilizer is important as a supplement because it contains Nitrogen which plays role as a macromolecule for hyphal formation (Saidah, 2019). Recent research showed that supplementation of NPK into liquid media can achieve conidial biomass up to 5.1 g/L (Mishra, 2012).

Another potential supplementation is coconut water. It is considered potential for improving *B. bassiana* because it is rich in nutrition (glucose & minerals) (Purnima, 2012; Indriyanti et al., 2021) and hormones (auxin, cytokinin, and gibberrelin) (Gopal et.al**,** 2018). Recent research showed the potential of coconut water as an additive supplementation for cultivating *B. bassiana* can achieve blastospore density by $3.8x10^7$ (Purnima et al., 2012) and 2×10^8 (Mona et al., 2016). Based on this problem, this research was conducted to 1) Analyze the physical characteristics of *the B. bassiana* colony and physical changes that occurr in the liquid media, 2) Analyze nutritional changes of the liquid media, and 3) Analyze the pH level changes of the liquid media. This study is the combination of each liquid media (potato sugar extract) supplemented with NPK 1% and or coconut water. This research is expected to provide additional information about *B. bassiana* cultivation on liquid media.

METHODS

This research was conducted in Biology Laboratory Mathematics and Science Faculty, from January until April 2022. *B. bassiana* was obtained from BPTPHP Salatiga in a solid form grown on PDA (Potato dextrose agar) medium. This research contained six treatments with four times repetition. The treatment groups of this research are:

1) distilled water (150 mL) + *B. bassiana* (150 µl);

2) Potato Dextrose Broth (PDB) (150 mL) + *B. bassiana* (150 µl),

3) Potato sugar extract (PSE) medium (150mL) + *B. bassiana* (150 µl);

4) PSE (132mL) + NPK 1% (18 mL) + *B. bassiana* (150 µl);

5) PSE (133.5mL) + coconut water (16.5 ml) + *B. bassiana* (150 µl);

6) PSE (115.5mL) + coconut water (16.5 ml) + NPK 1% (18 mL) + *B. bassiana* (150 µl).

Beauveria bassiana suspension was dissolved into 9 ml of sterile distilled water before the inoculation into the liquid media. All samples were incubated in a 250 ml Erlenmeyer flask. The entire process of this research is presented down below.

Preparation of Potato Dextrose Broth (PDB) Medium

Potato dextrose broth (PDB) powder was prepared in 24 grams. Then, PDB powder was added to 976 ml of sterile distilled water in a pan while heated in low heat for at least 20 minutes. The mixture was then cooled and poured into an Erlenmeyer flask for 150 ml. The medium was then sterilized using autoclave at 121°C and 15 psi for 15 minutes.

Preparation of Potato Sugar Extract (PSE) Liquid Medium

Two hundred grams of peeled and washed potato were prepared, then cut into small cubes. Next, the potato cubes were boiled with 1000 ml of sterile distilled water in a pan with medium-low heat until it was half tender. Then, they put out the pan and leave the potato liquid only. The potato liquid was reheated and mixed with 20 grams of sugar until it was boiled (15 minutes). If the volume of this solvent decreased by half, sterile distilled water was added until this mixture reached 1000 ml. As much as 150 ml of potato liquid was poured into a 250 mL Erlenmeyer flask.

The next step was to sterilize the PSE liquid in the autoclave at 121°C and 15 psi for 15 minutes.

Preparation of Potato Sugar Extract (PSE) + NPK 1%

This medium consisted of PSE and NPK 1%. The PSE used as a stock solution that had been made previously. NPK 1% was made by mixing 1 gram of NPK fertilizer into 99 mL of sterile distilled water. Then, 132 mL of PSE (88%) was mixed with 18 mL NPK 1% mixture (12%). The mixture was then sterilized using the autoclave at 121°C with 15 psi for 15 minutes.

Preparation of Potato Sugar Extract (PSE) + Coconut Water

This medium consisted of PSE and coconut water. The coconut water used in this study was from a young coconut. The preparation was started by filtering the coconut water. As much as 100 ml of coconut water was then sterilized using the pasteurization method at 60-70ºC for 15 minutes. Next, 133.5 ml of PSE (89%) was mixed with 16.5 ml of coconut water (11%) using sterile Erlenmeyer flask. The following step was the sterilization of the mixture using an autoclave at 121°C with 15 psi for 15 minutes.

Preparation of Potato Sugar Extract (PSE) + NPK 1% + Coconut Water

This medium consisted of PSE, NPK 1%, and coconut water. The preparation was started by mixing 115.5ml PSE (77%), 18ml NPK 1% (12%), and 16.5 ml coconut water (11%). The solution was then sterilized using the autoclave at 121ºC for 15 minutes.

Preparation Aquades Media

A stock of 1000 mL of distilled water is prepared first, then sterilized in the autoclave at 121°C at 15 psi pressure for 15 minutes.

***B. bassiana* Inoculation and incubation into Liquid Media**

The inoculation of *Beauveria bassiana* into the liquid media consisted of two main steps i.e. making the fungal suspension and inoculating them into the media. The fungal suspension was made by mixing 1 gram of *B. bassiana* spore with 9 ml of distilled water inside the reaction tube. Then, the inoculation process was performed by mixing 0.15 ml of *B. bassiana* spore suspension into 150µl of each liquid medium. *Beauveria bassiana* was incubated at 25-28ºC (room temperature) for 30 days.

Measurement of the pH Level of the Media

PH of each medium was measured before and after 30 days *of B. bassiana* incubation. The tool used to measure the pH level was a digital pH meter.

Analysis of Nutritional Content of the Media

Nutritional content of each liquid medium was tested before and after 30 days *B. bassiana* incubation. The test was conducted at Biology Laboratory, Mathematics and Science Faculty, UNNES. Nutritional measurements performed consisted of carbohydrate, lipid, and protein tests.

Analysis Data

Physical characteristics of *B. bassiana* colonies, physical and nutritional changes of the media, blastospore density and pH level of each medium were analyzed descriptively.

RESULTS AND DISCUSSION

Physical Characteristics of *B. bassiana* Colonies

Physical characteristics of *B. bassiana* colonies on liquid media after 30 days of incubation (shape, color, texture, and position) are shown in Figure 1.

In distilled water medium, there was no difference observed after 30 days of incubation. Meanwhile, in the PDB medium, the initial golden-yellow medium became turbid, and the color turned orange. The white *B. bassiana* mycelia started to appear on the surface of the medium on the 4th day of incubation. On the 10th day, the fungi had covered the entire surface, and powdery conidia were observed on the 25th day after inoculation.

PSE medium that was initially clear with yellowish color became yellowish-turbid. *B. bassiana* started to appear on the 5th day after the inoculation. The fungal colony initially grows on the surface of the medium, but then settles at the bottom of the medium on the 30th day of incubation. In PSE medium supplemented with NPK 1%, the medium that was initially white and turbid became yellow with fungal mycelia on the surface of the medium. The fungi started to appear on the 4th day of incubation. *B. bassiana* white, round, and smooth colonies were then fully covered on the medium surface after 5 days of inoculation. On the 20th day of incubation, two big layers of fungi started to appear, and on the 30th

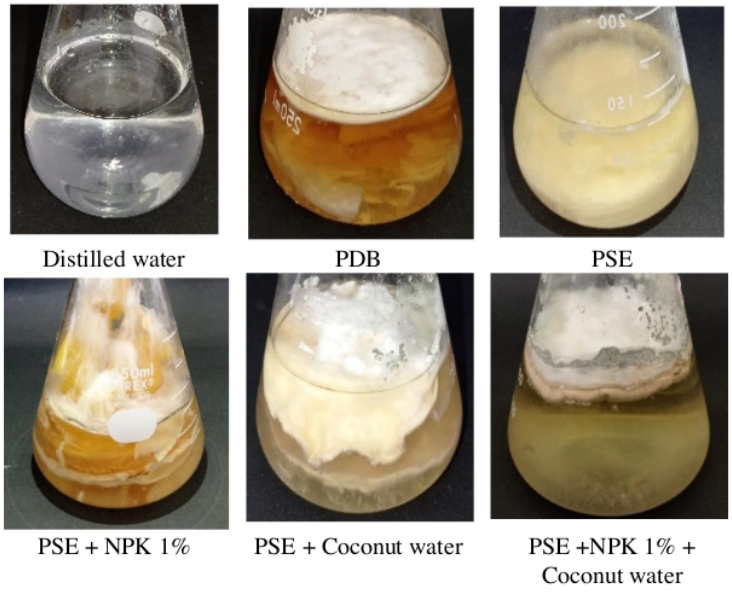

Figure 1. *Beauveria bassiana* growth on liquid media

Note: PDB (Potato Dextrose Broth); PSE (Potato Sugar Extract); NPK (nitrogen, phosphate and potassium); Coconut water.

day, the colonies became filamentous.

In the PSE medium supplemented with coconut water, the medium had changed from yellowish white to turbid yellow. *B. bassiana* white, round, and smooth colonies started to appear on the 3rd day of incubation and fully covered the medium on the 5th day. On the 20th day of incubation, the colonies became thicker, and rough, and made numerous layers. At the end of observation (30th day) the colonies had made more layers. In PSE medium supplemented with NPK 1% and coconut water, the medium had changed from greyish yellow to turbid yellow. *B. bassiana* colonies started to appear on the 3rd day after inoculation with white, round, and smooth colony surfaces. On the 10th day, *B. bassiana* covered the surface of the medium and became thicker with the rough surface after 30 days of incubation. Growth of *B. bassiana* in most liquid media (except in distilled water) showed that this fungus has an opportunity to cultivate in the liquid media. There is a tendency that the media is best PSE + Coconut water media is best for *B. bassiana* growth.

The growth of *B. bassiana* started with the growth of little round spots of mycelia that become larger, thicker, and finally fulfill the surface of the media. The colony texture of *B. bassiana* in the liquid media started with a smooth surface after 5-15 days of inoculation and become rough and powdery on the 20th day after inoculation. This result showed that *B. bassiana* was already adept with liquid media to form spores. *B.bassiana* growth pattern in liquid media is also explained by Quiroz (2014) that in unstirred liquid media, the fungal mycelia and spores appeared on its surface. The observation of the fungal colony showed that the colony has milky white color and started to appear on the surface of the media. The colonies then tend to drop to the bottom of the media with the addition of new fungal mycelia on the surface of the media. *B.bassiana* cultivation on liquid media showed a special infectious organelle than in solid media (Mascarin et al, 2018). In fungal cultivation using liquid media, an infectious organelle called

Table 1. Nutritional content of liquid media before and after 30 days of *B. bassiana* inoculation

No	Sample	Carbohydrate		Protein		Lipid	
		Before	After	Before	After	Before	After
1.	Potato Dextrose Broth (PDB)	15.627	15.643	4.656	2.557	0.240	0.300
2.	Potato Sugar Extract (PSE)	14.831	15.559	4.191	2.484	0.260	0.260
3.	PSE + NPK 1%	15.011	15.561	4.613	2.894	0.280	0.200
4.	PSE + Coconut water	15.458	15.619	5.397	2.618	0.300	0.260
5.	PSE + NPK 1% + Coconut water	15.413	15.591	4.846	2.759	0.300	0.240

Note: PDB (Potato Dextrose Broth); PSE (Potato Sugar Extract); NPK (nitrogen, phosphate and potassium); Coconut water.

blastospore which is a yeast-like vegetative cell of *B. bassiana* was found.

Nutritional Content Measurement of the Liquid Media

Nutritional measurement was performed on liquid media before and after 30 days of *B. bassiana* inoculation. The test aimed to determine the most useful nutrition for the growth of *B. bassiana* based on the degrading score. The data are presented in Table 1.

Table 1 shows that the carbohydrate content tend to increase in all samples although in small amount. An increase in carbohydrate content is due to the presence of glucans (carbohydrate) from the fungal particle that dissolved in the samples. According to El Ghany (2016), glucan is glucose derivative linked with β-(1,3) or β-(1,6) that are able to provide rigidity for *B. bassiana* cell wall. The other bonds found in fungi are α-(1,3) and α-(1,4) that function as the matrix of the fungi (El Ghany, 2016).

According to the result of protein measurement, it can be seen that the protein content in all media decreases after 30 days of *B. bassiana* inoculation. The highest decrease was obtained from PSE+coconut water treatment (from 5.397 to 2.618). This condition occurred since protein has a significant role in the metabolism of *B. bassiana*. Protein is the source of nitrogen that is also expressed in the form of permeases and catabolic enzymes. Protein plays a role in the body's defense system and interactions against pathogens (Mascarin et al., 2018), induces the sporulation process (Quiroz, 2014), has a positive effect on the growth of fungal mycelia (Bhadauria, 2012), and plays a role in the formation of organelles needed for the fungal apical growth (Pramesti, 2014).

Based on the lipid measurement (Table 1), 3 of 5 samples show a decrease in lipid content. Lipid is used in fungal metabolism to form glycerophospholipids, as a protector against environmental stress (Liu et al., 2015), and involves in the process of pathogenicity against the host insects (Yadav et al., 2013).

Measurement of pH Media

Measurement of pH media aimed to identify the difference in pH level before and after 30 days of *B.bassiana* cultivation. The results of the measurement are presented in Table 2.

According to Table 2, most treatments show an increase in pH level after 30 days of inoculation. Alkalosis reaction is suspected to be one of the causes of this condition. Alkalosis occurs due to the accumulation of bicarbonate ions in cells' internal environment (Brinkmann & Sharma, 2021 An increase in pH level can also be caused by an

Table 2. pH level of liquid media before and after 30 days of *B. bassiana* inoculation

Sampling Time	pH Media					
	Distilled water	PDB	PSE	PSE+NPK 1%	PSE + Coconut water	PSE+NPK 1% + Coconut water
Pre-inoculation	6.8	6.2	6.5	6.5	6.4	6.4
Post-inoculation	6.8	7.0	6.6	6.8	6.9	6.8

Note: PDB (Potato Dextrose Broth), PSE (Potato Sugar Extract), NPK (nitrogen, phosphate and potassium), Coconut water.

increase in ammonia level due to protein catabolism (Vylkova, 2017). In this case, ammonia can be removed in the form of ammonia itself or in the form of urea. An increase in pH level is important for the pathogenicity of *B.bassiana* against the host cell. This condition allows *B. bassiana* to secrete cuticle-degrading protease enzymes as a response to the host's cuticle that has high acidity (Jin et al., 2010).

Coconut water contains complex organic materials including free amino acids, vitamins, minerals, and sugar that significantly affect blastospore growth and even can maintain a shelf life of up to 9 months after packaging and storage (Mascarin et al., 2018 Moreover, coconut water also contains some important growth elements such as inorganic ions (potassium and sodium), 20 mg/ml sugar (sucrose, glucose, and fructose), hormones (auxin, cytokinin dan gibberellin), vitamin such as ascorbic acid, lipid, amino acid, organic acid, and minerals (Gopal et al., 2019). There were some difficulties in the measurement of *B. bassiana* blastospore density, because the fungal mycelia grow on the surface of the media forming a thick mass that makes it difficult to calculate the blastosopra density.

CONCLUSION

The physical characteristics of *B. bassiana* cultivated in liquid medium are white color, powdery conidia texture and located on the surface of the medium. Moreover, the medium shows different physical appearance and nutritional content before and after 30 days of incubation. There are increases in carbohydrate and decreases in protein and lipid as well as an increase in pH level 30 days after *B. bassiana* inoculation. The results of this study need to be followed up using a larger scale *B. bassiana* cultivated in liquid medium.

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