KnE Life Sciences
PGPR 2021
First Asian PGPR Indonesian Chapter International e-Conference 2021
Volume 2022

Research article

Conidia Production of Beauveria Bassiana in Solid Substrate Fermentation Using a Biphasic System

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Abstract.
Beauveria bassiana is an important entomopathogenic fungus that is widely used as a bioinsecticide around the world. Conidia production is a critical step in the production of high-quality bioinsecticide. This study investigated three liquid culture mediums and five combinations of solid substrates to enhance conidia production by *B. bassiana*. The fungus was isolated from infected insects in the cocoa plantation of PT. Perkebunan Nusantara XII in Kediri, East Java, Indonesia. The three culture mediums were malt extract broth (MB), potato dextrose broth (PDB), and yeast and malt extract broth (YMB). Five combinations of solid substrate were used: 100% rice, 100% maize, 75%:25% rice:maize, 50%:50% rice:maize, and 25%:75% rice:maize. The biphasic system was used in this study, in which the fungus was first grown under submerged conditions and then was allowed to conidiate in solid-state conditions. The data showed that PDB was the optimum culture medium to produce blastophore and beauvericin, the active compound that acts as a mycoinsecticide. In the selection test, 100% rice was the optimum solid substrate to produce high amounts of conidia, and the consistency and production tests yielded the same results, with conidia counts of 1.93x109, 1.78x109, and 2.08x109, respectively. In a rice storability test, *B. bassiana* conidia numbers remained stable for up to 105 days of storage at room temperature.

Keywords: Beauveria bassiana, culture medium, solid-substrate, conidia, biphasic system

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Published 07 June 2022

Publishing services provided by Knowledge E

Selection and Peer-review under the responsibility of the PGPR 2021 Conference Committee.

1. Introduction

The application of intensive traditional agriculture worldwide has raised problems due to the massive use of synthetic insecticide. The number of insects that developed resistance to chemical treatments was significantly increased [1], [2], as well as the numerous health problems of the exposed populations [3], [4]. Consequently, the innovation considering procedures and techniques to produce an environmentally friendly

insecticide based on microorganisms as biocontrol agents (BCAs) is also gained attention for crop protection against pests. Entomopathogen fungi (EF) are recognized as BCA with essential properties to combat insect pests with selective activities [5]. The formulation and production of this type of bioinsecticide are already well-established since the market for this product is also widely open [6]–[8].

Beauveria bassiana (Balsamo) Vuillemin, is a well-known EF and its role as BCA is already proven because of its ability to infect a different insect population in almost all insect orders [9]. The genetic variability of this fungus is high, which allows it to distribute throughout the world from temperate to tropical areas. This EF worldwide distribution is due to its adaptability to changing environmental conditions [10]. *B. bassiana* has exclusive properties besides its performance as an EF. This fungus can also grow inside plant tissues as an endophyte [11], have antagonistic activities against plant pathogens [12], can colonize the rhizosphere, and can live saprophytically in the soil [13], [14].

Aerial conidia as propagules are produced by *B. bassiana* in which under natural conditions has a function for dispersal and infection [15]. The flooding inoculation method is commonly used to apply *B. bassiana*, which sets a condition for propagules to interact with insects and the environment then starts an infective process. The requirement of this ideal condition is the propagules should be at a high concentration so that the interaction probability among fungi, insects, and the environment can be increased. The purpose of increasing yields of infective propagule from *B. bassiana* has been developed by applying new techniques and culture procedures.

Different methodologies were applied to produce a high amount of *B. bassiana* spores. Both liquid and solid mediums can be used as a substrate for the mass production of these spores. Two different spores can be formed depends on the medium type. Conidia are produced in a solid substrate (aerial conidia), while blastospores are produced in a liquid medium. The proportion of carbon and nitrogen source on the medium can induce *B. bassiana* to form conidia under submerged culture [16]. Conidia can also be produced in a biphasic system, in which fungus is first grown under submerged conditions to produce metabolic active blastospores and then allowed to conidiate in solid-state conditions [17]. The main difference between conidia and blastospore is their hydrophobic and hydrophilic properties. Aerial conidia are more hydrophobic, making these types of spores more suitable for oil formulation, while blastospore are hydrophilic, which has a disadvantage since their viability will lose in a relatively short time during storage [18]. Therefore, solid substrate fermentation is considered as a proper system for mass production of conidia from *B. bassiana*.

Substrate selection for the fermentation process has to be determined to produce a high number of conidia. This fungus can be cultivated in the synthetic liquid mediums which are commonly used in the laboratory [19] or using defined mediums with an adjusted C/N ratio from waste products [20]. As a solid substrate, rice grain has been selected as the most common substrate for the production of conidia [21], [22]. Rice has characteristics such as a proper C/N ratio, cheap, worldwide availability, uniform in size and shape, easily hydrated, and has the best structural integrity after being colonized by fungi. However, using a mixture between rice and agro-industrial wastes or other grains can also be considered to give an added value [17].

Our laboratory has a local isolate of *B. bassiana*, isolated from infected insects in the cocoa (*Theobroma cacao*) plantation of PT. Perkebunan Nusantara XII, in Kediri, East Java, Indonesia. This present study investigated a biphasic system to produce maximal conidia production by these local *B. bassiana*. Three different liquid culture mediums were selected to find the most suitable mediums to produce blastospore and beauvericin in submerged culture, followed by selecting a solid substrate using five different rice and maize grain combinations. Consistency and production test were done to prove that the most optimal substrate in the selection step gave the consistent conidia number. Furthermore, the quality of conidia in the dried powdered solid substrate was quantified during storage at room temperature for 105 days.

2. Methodology

2.1. Microorganism and submerged culture conditions

The morphological criteria described by Lacey [23] were used to identify the isolated fungus, and these fungi were identified as *B. bassiana*. Potato Dextrose Agar (PDA) was used to maintain the stock and working culture of *B. bassiana* at 28^{o}C. Fungal blastophores and beauvericin production were studied in the submerged culture at 28^{o}C on a rotary shaker at 160 rpm for 6 days. Six days old of working culture were prepared as inoculum by taking a full colony with a cork-borer. One hundred millilitres of different culture mediums in 250 mL Erlenmeyer flask were inoculated aseptically with three agar pieces with 1 cm in diameter fully grown by *B. bassiana*.

2.2. Selection of liquid culture medium

For quantification of blastospore number and beauvericin content, *B. bassiana* were cultivated in three different liquid culture mediums, which were Potato Dextrose Agar (PDA), Malt Extract Broth (MB), and Yeast and Malt Extract Broth (YMB). Compositions of 1 liter of every medium were: infused potato, 200 g; and dextrose, 20 g for PDB, malt extract base, 6 g; yeast maltose, 1.8 g; dextrose, 6 g; and yeast extract, 1.2 g for MB, and malt extract base, 3 g; yeast extract, 3 g; peptone, 5 g; and dextrose, 10 g, for YMB.

Beauvericin content was determined as an additional indicator to select the most suitable liquid culture for blastophores production by *B. bassiana*. Mycelial mass, including blastophores, was separated from the medium by filtration through filter paper using a vacuum pump, followed by air-dried at room temperature. A method developed by Moretti *et al*. [24] with modifications were used for beauvericin extraction procedure, and quantification of beauvericin content was done using High-Performance Liquid Chromatography (HPLC) following the protocol described by Logrieco *et al*. [25] with modifications. Detailed modifications for beauvericin extraction and quantification of beauvericin content from fungal samples developed by Roswanjaya *et al*. [19] were applied in this research.

2.3. Solid-substrate selection for conidia production

Five combinations of solid substrates were used to cultivate *B. bassiana*. The detail combinations of each mixture were: 100% of rice, 100% of maize, rice:maize (75%:25%), rice:maize (50%:50%), and rice:maize (25:75%). A clean-washed 100 grams of each mixture was transferred into a 250 mL Erlenmeyer flask, moistened, and autoclaved at 121^{o}C for 20 min. A biphasic fermentation system was applied, with the fermentation conditions were 40% moisture content (v/w), 25^{o}C culture temperature, the inoculum concentration of 10^{7} conidia/g wet solid substrates, 10% inoculum size, and 14 days incubation time [26]. The culture was shaken every two days to well-distribute the fungus growth on the solid substrate.

Fourteen days old conidiated substrates were harvested and dried using a tray system at 35^{o}C until the total weight was constant. The dried substrate was ground to form a powder state and sieved through 3 sets of sieves with a mesh size of 18 (1 mm), 35 (0.5 mm), and 60 (0.25 mm) [27]. The powder containing conidia, passing through all three sieve sizes, was stored before further quantification. The most optimum substrate for conidia production was selected based on the highest viability number of conidia.

2.4. Consistency and production test

The consistency test aimed to repeat the usage of the most optimum substrate to reconfirm the conidia number resulting from the selection test. The fermentation condition and processing steps were similar to that test. The consistency of the selected substrate in producing high viable conidia was done by comparing the conidia number between selection and consistency tests.

In the production test, a larger amount of optimum substrate was used to cultivate *B. bassiana*. Unlike the selection or consistency test, autoclavable plastic bags were used in the production test instead of the Erlenmeyer flask. One kilogram of substrate was clean-washed, moistened, autoclaved, inoculated and fermented with the same condition as the two tests before. The fermented substrates were gently hand-shaking every 24 hours. The dried substrate was then ground, sieved and viable conidia numbers were counted.

2.5. Conidia quality evaluation during storage

Conidia quality was evaluated based on the viable conidia number during storage for some intervals of time. Twenty grams of conidia powder were put in the plastic clip then stored in the dark condition at room temperature. The conidia powder was sampled at 8-time points (0, 15, 30, 45, 60, 75, 90, and 105 days after storage) to evaluate conidia quality. The viable conidia numbers were counted at each time point, and the results were compared among all time points.

2.6. Blastospore and conidia counting

The indirect method was used to count blastospore or conidia in terms of viability or colony forming units (cfu). Those spores were allowed to germinate on the surface of solid agar media, and the number of germinated spores was calculated as the number of blastospore or conidia. One milliliter of liquid culture overgrown by *B. bassiana* or 1 gram of conidia powder was diluted with 9 mL of sterile distilled water. This suspension was vortexed for 30 seconds. Serial dilutions were conducted until 10^{10} dilution time, vortexing for 30 seconds at each dilution. A hundred microlliters of the fungal or conidia suspension from 10^{5} until 10^{10} dilution time were inoculated dropwise into the Petri disk containing solidified agar medium. The suspension was homogenized on the medium's surface using a spreader. After free water was evaporated, the Petri disks were closed,

sealed with parafilm and incubated for germination in the growth chamber at 25^{o}C for 24 – 48 hours.

2.7. Experimental design and statistical analysis

All experiments were arranged in a completely randomized design. The blastospore counting in this study was carried out in triplicate. However, the effects of the different liquid mediums on beauvericin content were carried out once without replication. Five replicates were carried out for each solid substrate combination, the quantification of consistency and production test, and the evaluation of conidia quality at 8-time points. We used R Studio ver. 1.4.1106 to conduct statistical tests. To compare the blastospore or conidia number among treatments, we tested the average of viable spore numbers for significance differences with ANOVA and a post-hoc Duncan test for pairwise comparison with a 95% confidence interval.

3. Result and Discussion

3.1. Blastospore production of B. bassiana in different culture mediums

In the selection of liquid culture medium experiment, we found that the blastospore of *B. bassiana* can be produced in all three mediums tested (Figure 1A). However, the blastospore numbers were different among the mediums after 6 days post-inoculation. PDB was the most suitable culture medium to induce blastospore production. The number of blastospores was 9.53x10^{8} blastospore/mL, while in the YMB, blastopores were found in the lowest number, 2.47x10^{8} blastospore/mL. The fungal growth and spore production, in general, were influenced by components of the nutrients in the medium, mainly by carbon and nitrogen. *B. bassiana* does not need complex carbon or nitrogen sources since the fungus can be well-grown on a medium containing simple carbon like sucrose or glucose, nitrogen, and mineral components [28]. In the composition of the YMB medium, carbon and nitrogen originated from two sources: dextrose and malt extract and yeast extract and peptone. These double carbon and nitrogen sources could induce *B. bassiana* to produce high biomass [19]. Mycelial mass as the indicator of fungal growth was not measured in this experiment, but Roswanjaya *et al*. [19] has reported that at 6 and 12 days post-inoculation, fresh mycelial biomass was highest induced on the YMB compared to the PDB. The clear different medium compositions

to induce high biomass or high blastospore production shows that specific nutrients compositions were needed for the growth or reproduction of the *B. bassiana*.

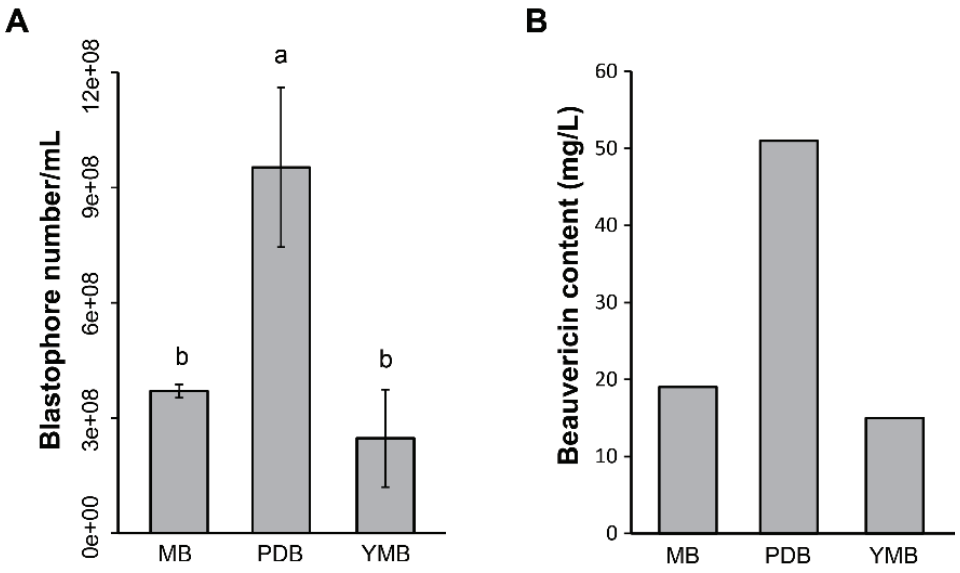

Figure 1: Blastospore production (**A**) and beauvericin content (**B**) of *B. bassiana* in three different culture mediums for 6 days of inoculation. Different letters above the bars indicate statistical significance ($p < 0.05$) as determined by the ANOVA test combined with Duncans's post-hoc test.

Beauvericin is the most important toxin produced by *B. bassiana* and has insecticidal activity [29]. In this experiment, beauvericin content was measured as an additional consideration to select the most optimal medium in inducing blastospore production. Beauvericin can be isolated from *B. bassiana* grown in all mediums (Figure 1B). The pattern of beauvericin content was similar to the effect of the medium on blastospore production. The highest beauvericin content was found in *B. bassiana* grown in PDB, followed by the fungus grown in MB and the YMB. The effects of specific carbon and nitrogen in the culture mediums on beauvericin content were hard to determine since only the complete mediums were used in this experiment. The selection of the basal medium with various defined carbon and nitrogen sources using response surface methodology can be used to determine the effect of each component [30]. Apart from that, our results show that PDB was not only optimal for blastospore production but also for inducing the highest beauvericin content.

3.2. Selection of solid substrate for conidia production

After 14 days of incubation, and processing substrates through drying, grinding, sieving, and conidia counting, the selection results demonstrated that 100% of rice was the best substrate for conidia production, followed by the combination of rice : maize (75%:25%),

rice : maize (50%:50%), and rice : maize (25%:75%) (Figure 2). Conversely, the result shows that 100% maize grains were less suitable for conidia production than others. The number of conidia for each substrate combination from the highest to the lowest were: $1.93x10^9$ conidia/g, $6.98x10^8$ conidia/g, $3.57x10^8$ conidia/g, $1.98x10^8$ conidia/g, and $1.62x10^8$ conidia/g of substrate, respectively. Our result was similar to experiments done previously to conclude that the most optimal substrate for the production of conidia by *B. bassiana* was rice grains [27], [31]–[33]. The different conidia number among the experiments was caused by the different fermentation conditions that applied. In contrast, our findings were opposite to Kreutz, *et al*. [34], which reported that optimal substrates for *B. bassiana* conidiation were maize, with the resulted total number of conidia was $3.27x10^9$/g. They did not use rice in their substrate selection, so we do not know the conidia production if rice was used as a substrate in their system. Moreover, the fermentation conditions were not detail mentioned in their report, makes a fair comparison could not be made.

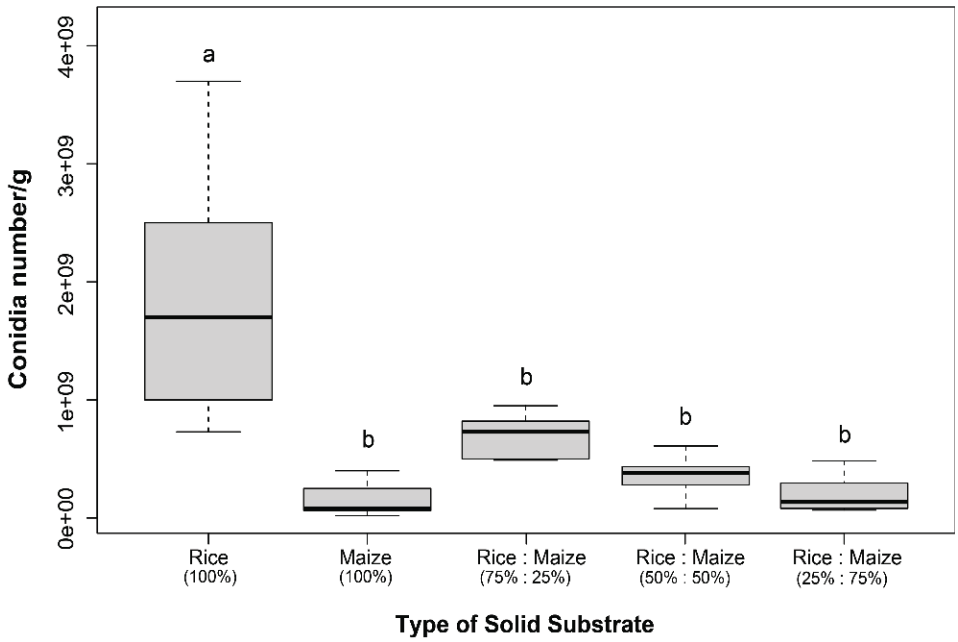

Figure 2: Conidia production on different solid substrate combinations at 40% moisture level, 25°C, inoculated with 10% inoculum containing 10^7 conidia/g wet substrate for 14 days. Different letters above the bars indicate statistical significance ($p< 0.05$) as determined by the ANOVA test combined with Duncans's post-hoc test.

The clear pattern observed from our result was the effect of maize grains on conidia production. The higher proportion of maize grains in the whole substrate, the lower number of conidia counted. As a substrate, rice and maize acting as both a carrier and nutrient sources. The nutrient compositions between rice and maize are different,

making the nutritional balances in those grains also different [34], affecting the fungal colonization and conidiation. The other condition that influences the fermentation process during conidia production is the physical characteristic of the substrates [27]. Maize grains have a thicker cell wall structure than rice, making this substrate harder to colonize and conidiate by *B. bassiana* even after autoclaved process [35], supporting our results which conidia number in only maize substrate was the lowest. Unlike maize cell structure, the thinner cell wall of the rice shows better hydration properties, which makes this substrate perfect for mycelial growth and fungal conidiation. The hydration properties also directly affected the structural integrity during colonization. In our observation, maize grains substrate or combination of the substrate containing maize grains shows weaker structure integrity during the fermentation process.

In this experiment, the selection using the proportion of rice and maize was aimed to increase nutrient concentration and enhance the substrate's porosity. An ideal substrate should maintain a proper porosity that supports its structural integrity during fermentation, which keeps the superficial area to volume (*A/V*) ratio higher [31]. Our result demonstrated that the substrates containing the rice and maize grains were not increasing conidia production because of the maize grains sizes, which reduce the *A/V* ratio in the total mixture. This fact suggests that other grains or products with better nutrition compositions and higher *A/V* ratio should be considered to be used together with rice.

3.3. Conidia production on consistency and production tests

Two different tests were applied to observe the consistency of rice in inducing conidia production as high as in the selection test. It is recommended to select a substrate that gives consistent results from batch to batch [31]. Our consistency test, which repeated conidia production using the same condition as in the selection test, shows a similar result on conidia number/g (Figure 3). However, compared to the selection test, the conidia number/g in the consistency test was slightly lower ($1.78x10^9$ compared to $1.93x10^9$ conidia/g). This slight reduction was no significant between these two tests based on statistical analysis. Furthermore, this result suggests that rice was a consistent substrate for conidia production by *B. bassiana*.

The second test also aimed to observe the consistency of rice as the most optimal substrate was the production test. Different from selection and consistency tests, in the production test, we used a larger volume of rice (10 times larger) and autoclavable plastic bags instead of an Erlenmeyer flask. In this test, a similar conidia number/g

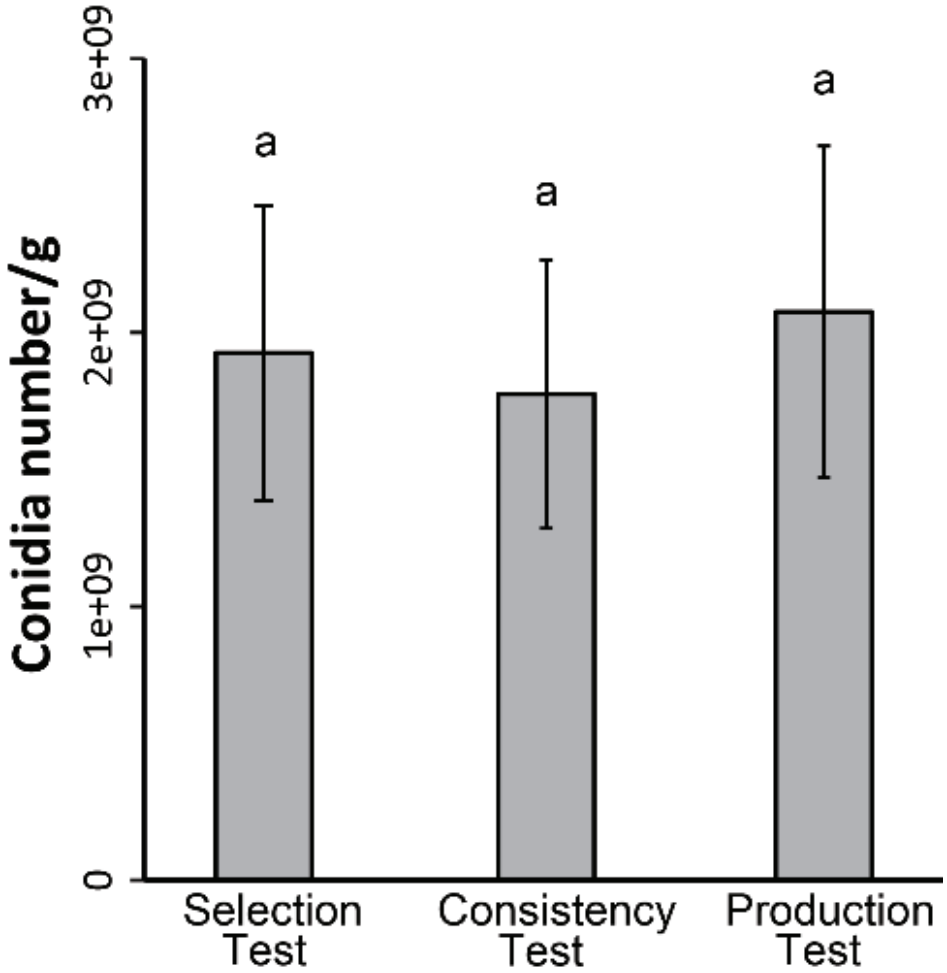

Figure 3: Conidia production of *B. bassiana* in selection, consistency and production tests. Different letters above the bars indicate statistical significance ($p< 0.05$) as determined by the ANOVA test combined with Duncans's post-hoc test.

resulted as in the selection and consistency test (Figure 3). The conidia number in this production test was $2.08x10^9$ conidia/g, a slightly higher from the previous two tests but not a significant difference according to the statistical test. Observation of detailed fermentation conditions in the production test indicates that plastic bags created more favourable conditions for fungal colonization and conidiation. This plastic bag system also provides better internal air circulation and higher heat removal [36], which induce an optimal level of conidiation.

Rice which was proven as the best substrate for *B. bassiana* conidia production in this research, has a high homogeneity in the sense of the particle size and geometry of the substrate. According to Membrillo *et al*. [37], both size and substrate geometry affect the fungal specific growth rate and is related to the production of the maximum enzyme involved in the degradation of the substrate. Moreover, simpler nutrition products resulted from substrate degradation, allowed maximal nutrients utilization by fungi and

affected growth and conidia production. From this experiment, the rice homogeneity can be confirmed as a factor that affects the homogeneous production of conidia resulted from these three different tests.

3.4. Storage stability of the B. bassiana conidia at room temperature

The viability of *B. bassiana* conidia stored at room temperature was assessed. The results showed that storage under room temperature, the viability of *B. bassiana* conidia declined gradually along 8-time points observed with no significant differences among values (Figure 4). After 105 days of storage, the number of viable conidia was $6.68x10^8$/g, decreased about 30% from the initial number at day 0. This suggests that conidia were still viable with a germination rate was 70%. A similar decreasing pattern of conidia viability was also reported by Sy, *et al*. [38], who observed the germination percentage of *B. bassiana* was reduced by 20-50% within 30 days of storage at 30^{o}C. In contrast, a high conidia germination rate, more than 85%, were retained in the samples stored at 4^{o}C for up to 24 months [39]. The germination rate was influenced by an interaction between storage temperature and storage time. The low temperature can keep conidia in the dormancy state longer and maintain the vigour of conidia, increasing conidia longevity [40].

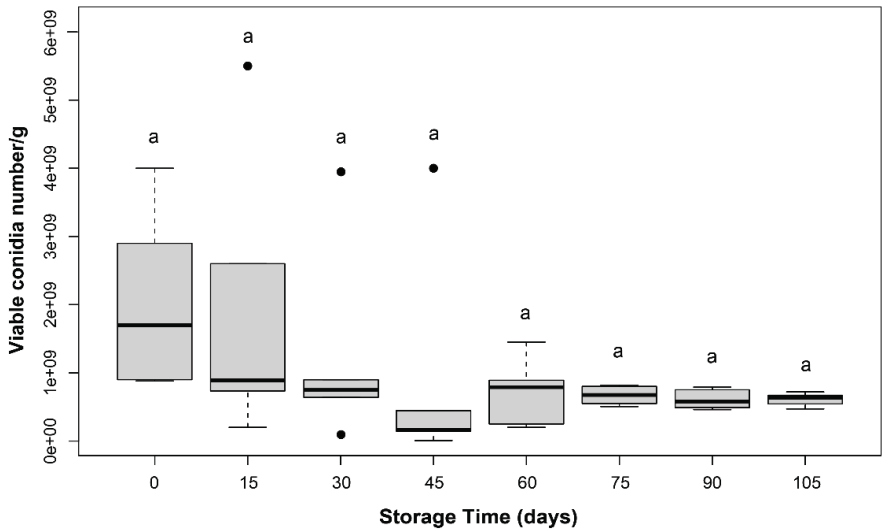

Figure 4: The viability of *B. bassiana* conidia at 8-time points at room temperature. Different letters above the bars indicate statistical significance ($p< 0.05$) as determined by the ANOVA test combined with Duncans's post-hoc test.

The conidia viability and germination speed are the most important properties for entomopathogenic fungi [41] that should be evaluated during storage. The loss of

attribute affecting the conidia effectiveness in a relatively short period has to be avoided since the minimum desirable shelf lives for biological insecticides are 6 months under controlled conditions [42]. In this present study, room temperature was used as a storage temperature which the relative humidity and constant temperature were uncontrolled. Those factors directly affect conidia's physiological changes during storage and can cause a decrease in its viability. The storage conditions and the use of additional components to maintain the conidia stability during the fermentation process or storage are suggested to be improved.

4. Conclusion

The biphasic system is the ideal two sequentially fermentation processes for conidia production of *B. bassiana*. PDB was the optimal liquid culture medium to produce blastospores in the submerged condition, whereas for the solid-state condition, rice was the best substrate to induce maximal conidia production. Moreover, rice as a substrate has already proven in resulting a consistent conidia number from batch to batch. Additionally, rice substrate also has the properties to keep a high number of viable conidia during storage up to 105 days. The substrate selection results obtained from this study may be useful for efficient conidia production as the first step in *B. bassiana* bioinsecticide formulation.

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